THE EVOLUTION OF ANTAGONISTIC SOCIAL INTERACTIONS IN BACTERIA: AN INVESTIGATION OF SPITE, COMPETITION, AND VIRULENCE IN *XENORHABDUS SPP*

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THE EVOLUTION OF ANTAGONISTIC SOCIAL INTERACTIONS IN BACTERIA: AN INVESTIGATION OF SPITE, COMPETITION, AND VIRULENCE IN *XENORHABDUS SPP*

This dissertation examines the evolution of a spiteful bacterial trait, bacteriocin production using insect-pathogenic bacteria in the genus *Xenorhabdus*. Bacteriocins are anticompetitor toxins produced by almost all known lineages of bacteria. Bacteriocin production is considered spiteful because it is costly to the producer; it also results in the killing of competiting strains of bacteria. Moreover, the costs of production are often too great to outweigh the direct benefits of competitor killing. Many costly traits in nature are maintained by being phenotypically plastic, and expressed only in environmental contexts where the benefits outweigh the costs. However, the role of competition-induced plasticity in the evolution of bacteriocin production has been largely untested. I empirically tested whether bacteriocin production is induced in response to the presence of non-self competitors, in a natural *Xenorhabdus* isolate (Chapter 1). Surprisingly, I found no evidence to support the plasticity hypothesis. This result was particularly puzzling because bacteriocin production in gram-negative bacteria like *Xenorhabdus* is often considered a 'suicide mission' where cell lysis is required for toxin release. Why would bacteriocin-producing cells commit 'suicide' in the absence of any competitors? To address this question, I used a modeling approach and examined the simplest conditions necessary for costly bacteriocin production to invade a metapopulation of faster-growing, sensitive cells (Chapter 2). Results show that bacteriocin release by lysis upon natural cell death can be sufficient for bacteriocin production to be favored; thus, competitor-induced plasticity is not necessary for bacteriocin production to be maintained.

Next, I examined the consequences of spiteful bacteriocin production on disease. Owing to their substantial growth costs, spiteful behaviors are predicted to reduce within-host pathogen growth and thereby pathogen virulence, which is the degree of damage a pathogen causes to its host. Consistent with predictions, I found evidence for reduced levels of bacteriocin production in experimentally evolved lineages that show greater virulence (Chapter 3). Finally, I investigated the evolution of bacteriocin resistance and showed that the incorporation of live, heterospecific competitors in conjunction with bacteriocin doses can reduce the emergence of bacteriocin resistance *in vitro* (Chapter 4). In the face of the current antimicrobial resistance crisis, these results provide strong proof-of-concept for a novel approach to impede the emergence of resistance against alternative antimicrobials such as bacteriocins. Taken together, this dissertation provides novel insights into the evolution and maintenance of bacteriocins in pathogenic bacteria, and attests to the importance of social interactions in shaping pathogen evolution.

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INTRODUCTION

The Evolution of Antagonistic Social Interactions in Bacteria : An Investigation of Spite, Competition, and Virulence in *Xenorhabdus spp.*

Social interactions can profoundly impact the fitness of organisms (Hamilton, 1964a; b; Frank, 1998). Growing evidence suggests that even simple unicellular microbes and viruses exhibit a diverse array of complex and sophisticated social interactions (Crespi, 2001; West *et al.*, 2007a; Strassmann *et al.*, 2011; Díaz-Muñoz *et al.*, 2017). Besides being fascinating in their own right, social interactions among microorganisms are of interest to biologists for a few distinct reasons. Microbial social interactions offer an excellent opportunity to test the generality of social evolution theory (West *et al.*, 2006, 2007a). Their short generation times makes microorganisms amenable to long-term evolutionary experimentation, and the ease of genetic manipulation further expands the scope of questions that can be addressed using these systems (West *et al.*, 2007a). Moreover, when these microbes are pathogenic, their social traits can have substantial impact on disease, and offer novel opportunities for disease management (Foster, 2005; Brown *et al.*, 2009; Leggett *et al.*, 2014). Owing to their potential for illuminating the evolution of social behaviors and their impact on disease, understanding the social lives of microorganisms, especially pathogens, has attracted considerable scientific attention over the past few decades.

A ubiquitously expressed social trait in bacteria, including pathogenic bacteria, is the production of costly, anticompetitor toxins called bacteriocins, which can kill cells of closely

related competitor strains, but do not kill clonemates of the producing cell (Reeves, 1965; Riley & Chavan, 2006). These ribosomally synthesized antimicrobial peptides exhibit a wide range of killing mechanisms including enzyme inhibition, nuclease activity, and pore formation in cell membranes (Riley & Wertz, 2002a; Riley & Chavan, 2006). Despite the competitive benefits of bacteriocin production, the synthesis and release of such large, proteinaceous toxins imposes high growth costs, and bacteriocin release often requires cell lysis and therefore, death, particularly in gram negative bacteria that lack a cell wall (Riley & Wertz, 2002b; Riley & Chavan, 2006). These negative fitness consequences on both the producer and the target competitor classify bacteriocin production as a spiteful trait (Gardner *et al.*, 2004; West *et al.*, 2007a). Despite such costs, bacteriocin production is ubiquitous, and almost all known lineages of bacteria and archaea produce at least one bacteriocin (Klaenhammer, 1988; James *et al.*, 1991).

In this dissertation I examine the evolution and maintenance of spiteful bacteriocin production, and its association with disease in natural isolates of the insect-pathogenic bacteria *Xenorhabdus* spp. This research is broadly motivated by three questions:

1. How is spiteful bacteriocin production maintained despite being so costly?
2. Does investment in spiteful bacteriocin production affect host-pathogen relationships?
3. Can we harness insights from this ubiquitous bacterial trait for disease management?

In the following sections, I summarize the specific contributions my dissertation makes towards addressing these questions. In Chapters 1 and 2, I examine the evolution and maintenance of

spiteful bacteriocin production using experiments and modeling. In Chapters 3 and 4, I examine how spiteful competition is associated with disease and may influence disease management strategies. This work advances a growing body of literature, which demonstrates the importance of social interactions in shaping, and being shaped by, evolution in the microbial world.

Chapters 1 and 2 : The evolution and maintenance of spiteful bacteriocin production

The evolution of spiteful behaviors poses an important challenge to evolutionary theory (Hamilton, 1970). How can a trait that decreases the fitness of both actors and recipients be maintained by selection? I examine this question by studying bacteriocin production, which is a premier example of spiteful behavior in nature (West *et al.*, 2007a). Theory and experiments suggest that the competitive environment is key for the maintenance of bacteriocin-producing lineages (Chao & Levin, 1981; Gardner *et al.*, 2004; Inglis *et al.*, 2009). Specifically, theory predicts that resource allocation to bacteriocin production is most favored when the producing lineage occurs at intermediate frequencies relative to a competing lineage (Gardner *et al.*, 2004). Consistent with this prediction, empirical work with the opportunistic pathogen, *Pseudomonas aeruginosa*, has shown that the relative fitness of a bacteriocin-producing lineage in competition with a bacteriocin-sensitive, competitor lineage, is the highest when the starting frequencies of the producer and sensitive strains is 1:1 (Inglis *et al.*, 2009). Thus, bacteriocin production is most beneficial when producers and competitors occur at comparable frequencies. As such, it is reasonable to hypothesize that phenotypically plastic bacteriocin production – in which bacteriocins are only released when competitors are present – may be favored by selection. This hypothesis remains largely untested although there is some evidence in *E. coli* for increased

bacteriocin release upon 'provocation' by competitors (Majeed *et al.*, 2011; Gonzalez *et al.*, 2018; Mavridou *et al.*, 2018).

In **Chapter 1** of my dissertation, I tested the hypothesis that bacteriocin production is phenotypically plastic and upregulated in the presence of non-self competitors *in vitro*. I conducted the work using natural isolates of *Xenorhabdus spp. Xenorhabdus spp.* are insect-pathogenic bacteria that have been a premier study system for the study of spiteful bacteriocin production in nature, and bacteriocin-mediated interactions are known to occur within the insect-host (Hawlena *et al.*, 2010a,b; Bashey *et al.*, 2012, 2013). To test whether bacteriocin production in these bacteria is induced in response to the presence of non-self competitors, the competitive environment of a bacteriocin producing strain (*X. koppenhoeffri*) was manipulated by growing the producer strain either in pure culture or in the presence of equal initial frequencies of a non-self strain. The competitor strain was derived from the producer's sympatric, bacteriocin-sensitive competitor, *X bovienii*. Bacteriocin extracts were collected at four different time points over the course of *in vitro* growth, and the inhibitory activity of the bacteriocin extracts was measured using a quantitative growth inhibition assay. Surprisingly, the results provided no evidence for increased bacteriocin production in the presence of the competitor (Bhattacharya *et al.*, 2018). Bacteriocin production was detected during late growth stages regardless of the competitive environment with no quantitative differences in the inhibitory activity of bacteriocins between the treatments. These results were puzzling.

Bacteriocin production and release is widely described in the literature as a 'suicide mission' for producing cells, especially gram negative bacteria, in which release requires cell

lysis (Nedelcu *et al.*, 2010; Mitri & Foster, 2013). It is frequently assumed that individual producer cells lyse and die for the sole purpose of releasing bacteriocins. If that is true, then the results of chapter 1 are even more puzzling. Why would producer cells commit 'suicide' to release bacteriocins in the absence of competitors? To understand if 'suicides' are indeed necessary for bacteriocin-mediated competition to be effective, Curt Lively and I developed a new model that examines the simplest conditions necessary for costly bacteriocin production to be favored. This model is described in **Chapter 2** of this dissertation. The model incorporates explicit demographic details and investigates the invasion of a rare, bacteriocin-producing mutant in a metapopulation dominated by a faster growing sensitive strain. Results demonstrate that bacteriocin release by lysis upon natural cell deaths, rather than additional 'suicidal' release, can be sufficient for a rare producer strain to invade a meta-population of faster-growing sensitive competitors. Further, competition-induced, plastic bacteriocin release can promote producer-invasion at increasingly global scales of competition, but the benefits of plasticity are lost if scale of competition is local. We argue that for pathogens like *Xenorhabdus spp.*,whose dispersal (or transmission) is numerically limited by vectors, the scale of competition is local, thus reducing selection for plasticity.

Taken together, these chapters investigate the largely untested role of phenotypic plasticity in the maintenance of spiteful bacteriocin production. Model results demonstrate that bacteriocins released by natural cell deaths can be sufficient for bacteriocin production to persist and that plasticity isn't necessary for the maintenance of spiteful bacteriocin production. These results may call into question whether bacteriocin production is indeed spiteful at the level of the individual producing cell as commonly assumed. Bacteriocin production is considered spiteful

under the assumption that the individual producing the bacteriocin sacrifices itself for the specific purpose of killing competitors. However, if the individual cells that release bacteriocins only do so upon natural death, the trait may not be spiteful in the same way as previously thought. Taken together the results of chapters 1 and 2, challenge the commonly made assumption that bacteriocin production is a 'suicide mission' and offer a new way of thinking about the maintenance of costly bacteriocin production

Chapters 3 and 4: Spiteful bacteriocin production and disease

Increasing evidence suggests that social interactions within pathogenic populations can have important consequences on disease (Leggett *et al.*, 2014). Cooperative bacterial traits such as biofilm formation and public goods production are directly associated with pathogens' ability to establish in new hosts and influence the degree of damage caused to the host, *i.e.* virulence (Koutsoudis *et al.*, 2006; Köhler *et al.*, 2009; Strateva & Mitov, 2011; Pollitt *et al.*, 2014). Within-host competitive interactions also have myriad effects on disease virulence. Increased competition for resources may select for faster pathogen growth resulting in increased virulence during mixed infections relative to single infections (de Roode *et al.*, 2005). In contrast, spiteful competitive interactions during mixed infections can reduce pathogen densities and select for reduced virulence (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Inglis *et al.*, 2009). By shaping pathogen growth and disease virulence, social traits in pathogens play a central role in pathogen evolution (Buckling & Brockhurst, 2008).

In **Chapter 3** of this dissertation, I examined the relationship between virulence and spiteful bacteriocin production in the insect pathogenic bacterium, *Xenorhabdus nematophila*.

Theory predicts a negative correlation between virulence and bacteriocin production because faster growth is associated with increased virulence, while bacteriocin production is costly and reduces pathogen growth (Gardner *et al.*, 2004). Consistent with this theory, experiments have shown that spiteful competition during mixed infections can result in reduced virulence (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Inglis *et al.*, 2009; Bashey *et al.*, 2011). However, very few studies have examined if the evolution of virulence and spite are linked within pathogen populations. Garbutt *et al.* (2011) found that the evolution of *Bacillus thuringiensis* lineages under mixed infections resulted in selection for reduced virulence and increased antagonism. The increase in antagonism occurred without evolution of increased growth rates, indicating that the antagonism was likely bacteriocin mediated. Nonetheless, whether and how virulence evolution affects levels of bacteriocin production is currently unclear.

In **Chapter 3,** I investigate the evolutionary interplay between virulence, growth, and bacteriocin production in *Xenorhabdus nematophila* populations. Using experimentally evolved lineages that show faster host-killing (Morran *et al.*, 2016) and thus, increased virulence, we asked whether increased virulence was associated with changes in levels of bacteriocin production and growth. Consistent with theoretical predictions, we found a significant decrease in bacteriocin production among the more virulent lineages relative to their ancestral population. The more virulent lineages also showed faster growth rates relative to their ancestor. Comparing the levels of bacteriocin production and growth across all the evolved and ancestral lineages revealed a significantly negative correlation, demonstrating the underlying growth costs of bacteriocin production. Overall, these results suggest that the evolution of greater virulence in these populations may have occurred via the evolution of faster growth at the expense of spiteful

bacteriocin production. These results offer some of the earliest insights into how bacteriocin production is affected by virulence evolution in pathogenic populations.

Intricate relationships between pathogen social traits and virulence can offer novel opportunities for disease management strategies to exploit (Foster, 2005; Brown *et al.*, 2009; Allen *et al.*, 2014b). Cooperative traits that can promote virulence are vulnerable to 'cheating' by mutants that do not invest in the cooperative trait themselves, but can spread through the population by exploiting the benefits of costly cooperation (Diggle *et al.*, 2007; Popat *et al.*, 2012). Using the ability of such 'social cheats' to disrupt cooperation in pathogenic populations may help reduce pathogen growth and thus, virulence (Sandoz *et al.*, 2007; Harrison *et al.*, 2017). Similarly, the trade-offs between virulence and spiteful competition as shown in Chapter 3 and by other studies (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Bashey *et al.*, 2011), suggest that maintaining selection for spiteful traits may help reduce virulence. Akin to using 'social cheats', the use of genetically engineered probiotics as therapeutics (Sola-Oladokun *et al.*, 2017) may help keep disease virulence low.

Competitive interactions among pathogens have also emerged as an important factor for managing the evolution of antimicrobial resistance (de Roode *et al.*, 2004; Wale *et al.*, 2017), which is among the most pressing public health challenges we currently face (World Health Organisation, 2018). Exposure to high antimicrobial doses over long durations can eliminate sensitive pathogens effectively (Ehlrich, 1913). However, by eliminating conspecific, antimicrobial-sensitive competitors, this approach also imposes strong selection favoring highly resistant mutants (Read *et al.*, 2011; Day & Read, 2016). Employing low antimicrobial doses can

maintain sensitive competitors to constrain the spread of resistant mutants (de Roode *et al.*, 2004; Kouyos *et al.*, 2014; Hansen *et al.*, 2017). However, this approach may fail if a healthy host cannot tolerate the levels of sensitive pathogens required for competitive suppression of resistance to be effective (Hansen *et al.*, 2017). Nonetheless, resource competition between conspecific antibiotic-sensitive and antibiotic-resistant mutants can prevent the emergence of resistance (Colijn & Cohen, 2015).

There is an urgent need to find alternative antimicrobials to replace conventional antibiotics that have been rendered ineffective as a consequence of resistance evolution (Allen *et al.*, 2014a). Bacteriocins are a proposed class of alternative antimicrobials because of their specific killing activity and ubiquitous production (Reviewed in Cotter *et al.*, 2013). However, to avoid a similar fate as antibiotics, it will be necessary to develop sustainable approaches of administering alternative antimicrobials like bacteriocins. In **Chapter 4**, I tested whether the principle of competitive suppression of resistance can be harnessed by using heterospecific competitors instead of relying on sensitive, conspecific competitors (Bhattacharya *et al.*, 2019). *In vitro,* proof-of-concept experiments were conducted to examine (a) how bacteriocin dose affects resistance-evolution in the lab, (b) whether incorporating live, heterospecific competitors in conjunction with antimicrobial doses can alter the outcome, and (c) could a bacteriocin-producing competitor alone be sufficient to achieve low target cell densities and resistance.

Our results show that exposure to a high bacteriocin dose can suppress total densities of sensitive cells effectively, but imposes strong selection for resistance. In contrast, resistance evolution is much reduced upon exposure to a low bacteriocin dose, but the total densities of

target cells remain high. Incorporation of a live heterospecific competitor strain in conjunction with the bacteriocin dose significantly reduced resistance evolution as well as total cell densities. The competitor strain used in the experiment wasn't sensitive to the bacteriocin used in the experiment, and it did not produce antimicrobials that affected the growth of the target strain. The combination of low bacteriocin dose and competitor had the lowest total as well as resistant-cell densities at the end of the experiment. This effectiveness was matched by using a bacteriocin-producing competitor directly as opposed to applying extracted bacteriocins in conjunction with a bacteriocin non-producing competitor. Taken together these provide strong proof-of-concept evidence to suggest that using live, heterospecific competitors in conjunction with antimicrobial doses can be an effective therapeutic strategy. For such a therapeutic strategy to work, the choice of the competitor strain will be very important. Bacteriocin-mediated interactions have been shown to drive the efficacy of many probiotic strains (Su *et al.*, 2007; Gillor *et al.*, 2009; Dobson *et al.*, 2012). Ongoing efforts to devise genetically engineered probiotic strains (Sola-Oladokun *et al.*, 2017) and harmless commensal bacteria may provide suitable candidates for consideration as therapeutic agents for the competitive suppression of resistance.

Taken together, Chapters 3 and 4 of my dissertation offer new insights into the intricate links between pathogen social traits and pathogen evolution. Our results demonstrate that spiteful bacteriocin production is negatively associated with virulence evolution, and show that competitive interactions can provide a force to counteract the evolution of antimicrobial resistance in bacterial pathogens. These results may have important implications for disease

management strategies and contribute to a rapidly-growing body of work in the field exploring exploitable links between pathogen social traits and disease.

To conclude, in this dissertation I investigated the evolution of spiteful bacteriocin production and its association with disease in the insect-pathogenic bacteria *Xenorhabdus spp.* My research addresses how such a costly trait can be maintained by selection, whether it is directly linked to disease evolution, and if it can offer insights for disease management. I examined the largely untested role of phenotypic plasticity in the maintenance of spiteful bacteriocin production (Chapters 1-2). Results from experiments and modeling suggest that bacteriocin production can be maintained without phenotypic plasticity in response to the presence of non-self competitors. Further, bacteriocin release as a consequence of natural cell deaths can be sufficient to maintain bacteriocin production. I also investigated the evolutionary link between virulence and spite and showed that the evolution of increased virulence is associated with reduced levels of bacteriocin production in the insect-pathogenic bacterium, *X nematophila (*Chapter 3). Finally, experiments in Chapter 4 examine the evolution of bacteriocin resistance and show that incorporating live, heterospecific comeptitors in conjunction with bacteriocin dose can significantly reduce the emergence of bacteriocin resistance *in vitro*. Taken together my dissertation attests to the power of bacterial social interactions to inform academic as well as applied pursuits in evolutionary biology.

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CHAPTER 1

Plastic responses to competition: Does bacteriocin production increase in the presence of non-self competitors?

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Abstract

Anti-competitor traits such as the production of allelopathic toxins can confer significant competitive benefits but are often costly to produce. Evolution of these traits may be facilitated by environment-specific induction; however, the extent to which costly anti-competitor traits are induced by competitors is not well explored. Here, we addressed this question using bacteriocins, which are highly specific, proteinaceous anticompetitor toxins, produced by most lineages of bacteria and archaea. We tested the prediction that bacteriocin production is phenotypically plastic and induced by the presence of competitors by examing bacteriocin production in the presence and absence of nonself competitors over the course of growth of a producing strain. Our results show that bacteriocin production is detectable only at high cell densities, when competition for resources is high. However, the amount of bacteriocin activity was not significantly different in the presence vs. the absence of nonself competitors. These results suggest that bacteriocin production is either (1) canalized, constitutively produced by a fixed frequency of cells in the population, or (2) induced by generic cues of competition, rather than

specific self/nonself discrimination. Such a non-specific response to competition could be favored in the natural environment where competition is ubiquitous.

Introduction

Competition is one of the most pervasive biological interactions, and it plays a key role in structuring ecological communities (Tilman, 1982). Anti-competitor strategies such as the production of allelopathic chemicals, can confer a competitive advantage to the producers, but such chemicals are often associated with high costs (Riley & Wertz, 2002b; Pintar & Starmer, 2003; Lankau, 2008). How are such costly competitive strategies maintained? Phenotypic plasticity may serve as one important mechanism for the maintenance of costly traits as the costs may be mitigated through conditional, environment-specific expression of the traits. For example, predator-induced morphological changes allow protection in the presence of a predator, but also allow for reduced costs in the absence of predation (Lively, 1986b; Baur *et al.*, 1991; Laforsch & Tollrian, 2004). Similarly, costly chemical defenses are well-documented to be induced by herbivory (Haukioja, 1991; Dicke & Hilker, 2003; Zangerl, 2003). However, the degree to which equally costly anti-competitor chemicals are induced by competition has not been well explored. To date, just a few studies have found evidence for competition-induced increases in the production of allelopathic compounds in some plants, algae, and bacteria (Lankau & Kliebenstein, 2009; Korgaonkar & Whiteley, 2011; Rasher & Hay, 2014).

Despite these examples of adaptive plasticity, not all costly traits will be phenotypically plastic. Phenotypically plastic genotypes need to maintain sensory and regulatory machinery required for detecting and responding to relevant changes in the environment, and may therefore

incur costs relative to non-plastic genotypes (reviewed in DeWitt et al. 1998; Murren et al. 2015). Other factors such as the timeliness of phenotypic response following environmental change, as well as reliability of environmental cues may impose limitations on the benefits gained from plasticity (Lively, 1986a; Moran, 1992; Padilla & Adolph, 1996; Scheiner & Holt, 2012). Further, environmental heterogeneity over relevant time scales for the trait in question is a key criterion for the evolution of adaptive plasticity (Bradshaw, 1965; Moran, 1992). Thus, whether a costly competitive trait is phenotypically plastic and induced by the presence of a competitor is likely to be determined by the interplay of multiple ecological and physiological parameters.

Bacteriocins provide an excellent opportunity to investigate whether costly anti-competitor chemicals are competitor induced. Bacteriocins are a class of allelopathic compounds produced by bacteria that can confer a competitive advantage by targeting and killing closely related competitors. A vast majority of archaea and almost every known lineage of bacteria are known to produce at least one bacteriocin (Klaenhammer, 1988; James *et al.*, eds, 1991; Riley & Wertz, 2002a). However, bacteriocin production is highly costly, requiring the lysis of producing cells in some species and the costs of production are often greater than the direct benefits gained through competitor killing (Riley & Wertz, 2002b; Riley & Chavan, 2006; Wloch-Salamon *et al.*, 2008). As such, bacteriocin production is considered one of the clearest examples of spite in nature whereby the producers incur a substantial cost and reduce the fitness of targeted competitor cells (Gardner *et al.*, 2004; West *et al.*, 2006). Theory predicts that resource allocation to bacteriocin production should be increased when the producing lineage occurs at intermediate frequencies with a competing lineage (Gardner *et al.*, 2004). Further, empirical

work has shown that the relative fitness of a bacteriocin-producing lineage is highest when the starting frequencies of the producer and sensitive strains is 50 : 50 (Inglis *et al.*, 2009). Therefore, it is reasonable to hypothesize that bacteriocin production is phenotypically plastic and increased in more competitive environments. This hypothesis remains largely untested.

The ability to sense appropriate changes in the environment is critical for phenotypic plasticity to be adaptive. Bacteria are known to respond to changes in their social environments (Kümmerli *et al.*, 2009; Rumbaugh *et al.*, 2009) and communicate with each other through diverse mechanisms including quorum sensing (Lerat & Moran, 2004; Waters & Bassler, 2005) and contact-dependent interactions (Gibbs *et al.*, 2008; Basler *et al.*, 2013; Ruhe *et al.*, 2013). Particularly relevant to bacteriocin production is the recently proposed 'competition sensing hypothesis' which posits that bacteria have evolved the ability to sense and respond to competition (Cornforth & Foster, 2013). A meta-analysis of bacteriocin regulatory pathways revealed that production is frequently regulated by stress-response pathways that are triggered by competition-related stressors such as nutrient stress and cellular damage. Conversely, bacteriocin production is rarely associated with stress response pathways that are sensitive to stressors like heat or osmotic stress, which are not competition related (Cornforth & Foster, 2013).

Here, we test whether bacteriocin production plastically increases in more competitive environments by examining bacteriocin production at different stages of growth in the presence and absence of nonself competitors. We test this prediction using natural isolates of *Xenorhabdus spp.* (Figure 1), which have been a key study system to investigate the maintenance of bacteriocin production in nature (Hawlena *et al.*, 2010a; b, 2012). *Xenorhabdus spp.* are insect-

parasitic bacteria that gain entry into an insect host by forming symbioses with entomopathogenic nematodes (Martens *et al.*, 2003; Herbert & Goodrich-Blair, 2007; Stock & Blair, 2008). Within the insect host, bacteriocin-mediated interactions occur and are known to affect the outcome of competition (Bashey *et al.*, 2012, 2013). We use field-collected isolates of *Xenorhabdus spp.*, which have a rich, competitive environment in nature where they may frequently, but not always, encounter nonself competitors within the insect host.

Materials and Methods

Experimental Design

A producer strain was grown alone (pure culture) or in the presence of a nonself competitor strain (mixed culture) and bacteriocin production was examined at multiple time points over the course of growth (Figure 2). Producer cell densities were measured at each time point examined. To measure bacteriocin production, filter-sterilized supernatants from the cultures were used in growth-inhibition assays, where the growth trajectory of a sensitive strain was used as a bioassay of the amount of bacteriocin in the supernatant. Specifically, the delay in growth of the sensitive strain induced by the presence of a bacteriocin extract was used to derive a metric of bacteriocin activity (Figure 2). As a negative control, cell-free supernatant from cultures of the competitor strain grown alone were run alongside the two producer treatments and were processed similarly at each time point during the bacteriocin-production phase of the experiment, as well as during the growth-inhibition assays (Figure 2).

Bacterial strains

The bacteriocin-producing clone used in this study was a natural isolate of *Xenorhabdus koppenhoeferi* (Kop46) which is known to produce a bacteriocin that can suppress the growth of a sympatrically isolated clone of *Xenorhabdus bovienii* (Bov59) (Bashey *et al.*, 2013). The competitor strain used in the experiment, Bov59R, was derived in the lab from Bov59. This strain was chosen because bacteriocin that is exposed to Bov59R cells maintains its inhibitory activity (Supplementary Materials S1). This was key for the current experiment to ensure that the inhibitory activity of the bacteriocin released by producing cells in the mixed treatment would not be subsequently altered by the presence of competitor cells. The natural isolate Bov59 which is sensitive to bacteriocin was used as the detector strain in the growth-inhibition bioassays. These strains of *X. koppenhoeferi* and *X. bovienii* are morphologically distinct (Figure 1), whereby Kop46 colonies appear maroon and Bov59/59R colonies appear blue on NBTA plates (nutrient agar supplemented with 0.0025% (w/v) bromothymolblue and 0.004% (w/v) triphenyltetrazolium chloride, pH = 8). In addition, Bov59/59R has a higher natural resistance to ampicillin than Kop46, thereby enabling further distinction on NBTA plates with 75μg mL^{-1} ampicillin. All cultures used in the experiment were derived from freezer stocks maintained at -80^0 C and streaked onto NBTA plates prior to each replicate.

Bacteriocin Production Treatments

To initiate each replicate, individual colonies of each strain were picked from freezer streaks and inoculated into 5ml LB media (Difco) in 20ml culture tubes. Cultures were grown overnight at 28^{o}C and then used to inoculate fresh LB broth to establish the three experimental groups. The mixed culture treatment was established by adding a fixed volume (50μl) each of the producer

Kop46 and the competitor Bov59R overnight cultures into 5ml LB. The producer alone treatment was established by adding 50µl of a Kop46 overnight culture and 50µl of LB to 5ml LB. Similarly, the negative control treatment was established by adding 50µl of the competitor Bov59R overnight culture and 50µl LB into 5ml LB. All culture tubes were incubated with shaking (120 rpms) at 28^{o}C.

Bacteriocin extracts were collected at four time points over the course of growth: 4 hours (early exponential), 6 hours (mid exponential), 12 hours (late exponential) and 24 hours (stationary phase). Cultures tubes were destructively sampled at each time point. A total of 10 producer and competitor colonies were examined at 24 hours and 6 colonies each were tested at the earlier time points. A small aliquot (100µl) was taken from each culture tube both immediately after setup and just before bacteriocin collection to calculate initial and final cell densities and to ensure the absence of contamination. These aliquots were diluted and plated on NBTA agar. Plates were incubated at 28^{o}C for 48 hours before colonies were counted. Cell density estimates confirmed that initial densities of producer and competitor cells in the mixed treatment were within the same order of magnitude (~10^6 c.f.u/ml) and the average frequency of producer cells was 0.61+/- 0.04 (mean +/- 1 S.E.M).

Bacteriocin extracts were collected by centrifuging cultures at 1620 G for 5 minutes and the supernatant was filtered through 0.45um filters (Acrodisc). This procedure allows the proteinaceous bacteriocin to pass through while eliminating any cells in the extract. These bacteriocin extracts were stored at 4^{o}C until use in the growth-inhibition assays. Growth-inhibition assays were performed within 7 days of bacteriocin extraction.

Growth-Inhibition Assay

The growth-inhibition assay was used to measure and compare the degree of growth inhibition induced by the application of bacteriocin extracts to a starting culture of bacteriocin-sensitive cells. Each bacteriocin extract was examined on 4 replicate wells in a 100-well plate reader (Honeycomb plate, Growth Curves USA). Each well contained a 200μl solution of a bacteriocin extract and starting cell culture in 1:5 ratio by volume. Varying cell densities of starting cell cultures were used (10^6, 10^5 or 10^4 c.f.u/ml) for bacteriocin extracts from different time points. The difference between the lag times induced by the negative control and bacteriocin samples increases with decreasing starting cell densities of the detector strain, facilitating the detection of bacteriocin-mediated inhibition (Supplementary Materials S2). Consequently, lower cell densities were used for the earlier time points where bacteriocin concentrations were low to facilitate detection of bacteriocin.

Every plate had internal control wells with un-inoculated media to rule out contamination. Culture growth in the wells was measured by a Bioscreen plate reader (Growth Curves USA) in which the plates were incubated at 28ºC with continuous shaking at medium amplitude. $O.D._{600}$ was recorded every 30 minutes for 24 hours.

Follow-up Growth Rate Analysis

To test whether the presence of Bov59R imposes competition on the producer cells, the growth rate of producer cells growing in the presence and absence of the competitor was estimated in an additional experiment. Paired replicates of the producer alone and mixed treatments were established using 10 different producer colonies and cell densities were estimated at 0, 6 and 24

hours from each tube. 100ul aliquots were used to make serial dilutions that were plated on NBTA plates to estimate cell densities. Growth rate of the producer over 6 and 24 hours was estimated for each tube as ln[producer density at final time point / producer density at 0hrs].

Statistical Analyses

The optical density measures for each well were used to compute lag times using the software program GrowthRates 2.1(Hall *et al.*, 2013). Lag time represents a measure of the delay observed before a culture moves into the exponential growth phase. GrowthRates 2.1 calculates lag time as the time point at which the extrapolated slope of the exponential phase line (lnOD vs time) meets the X-axis. Every well was manually examined for model accuracy in estimating lag-time.

The average lag time value of the four wells containing the same bacteriocin extract were used to calculate the relative lag time for each extract (Figure 2). The relative lag time induced by a given bacteriocin extract was calculated as the ratio of the average lag time value induced by the extract and the average lag time value induced by the corresponding negative control extract (extracts from competitor strain Bov59R pure cultures, Figure 2). A relative lag time value of 1 indicates no deviation from the negative control and relative lag time values significantly greater than 1 indicate inhibitory activity.

Relative lag times for bacteriocin extracted from pure cultures (Kop46 alone) and mixed cultures (Kop46 + Bov59R) were compared at each of the four time points. A mixed-model analysis of variance was conducted in SAS 9.4 with bacteriocin source (pure or mixed culture) as

fixed effect and 'colony identity' and 'experimental run' as random effects. Each experimental run was performed using one or two colonies of the producer and competitor.

Colony counts were used to compare the initial and final cell densities of the bacteriocin producer cells and to calculate producer growth rate (ln[producer density at final time point / producer density at 0hrs]) across treatments. Mixed model analyses of variance were used with 'treatment' (pure or mixed culture) as fixed effect and 'colony identity' and 'experimental run' as random effects.

For the follow-up growth rate analysis experiment, producer growth rates in the presence and absence of Bov59R were compared using mixed model analyses of variance with 'treatment' (pure or mixed culture) as fixed effect and 'colony identity' as random effect.

Results

Bacteriocin extracts were collected from the pure and mixed cultures at four distinct time points (4, 6, 12 and 24 hours). Their inhibitory activities were measured by examining the lag time they induced in the growth of the sensitive strain. Significant bacteriocin-mediated inhibitory activity was detected only at 12 and 24 hours (Figure 3, 95% C.I. do not overlap with 1). No bacteriocin-mediated inhibition was detectable at the earlier time points (Figure 3, 95% C.I. overlap with 1). There was no significant difference between the relative lag times induced by the extracts derived from pure cultures and mixed cultures at any of the four time points examined (Figure 3, 4 hours: $F_{1,10} = 0.06$, $p = 0.81$; 6 hours: $F_{1,10} = 0.20$, $p = 0.66$; 12 hours: $F_{1,10} = 0.29$, $p = 0.60$ and 24 hours: $F_{1,18} = 0.40$, $p = 0.53$).

We examined producer cell densities at the time of bacteriocin extraction to establish that bacteriocin activity was not confounded by differences in the total number of producer cells. Comparisons of producer cell densities between the pure and mixed cultures were not significantly different at any time point (Figure 4, 4 hours: $F_{1,10} = 0.84$, $p = 0.32$; 6 hours: $F_{1,10} = 1.28$, $p = 0.28$; 12 hours: $F_{1,10} = 0.39$, $p = 0.54$ and 24 hours: $F_{1,18} = 0.30$, $p = 0.59$). The density of competitor cells was reduced to, or below our detection limit of 10^5 c.f.u/ml at 4 hours, and 10^6 c.f.u/ml at 6, 12 and 24 hours. Comparable producer densities between treatments indicates that on a per cell basis, the producer cells did not differ in their bacteriocin production in response to the presence of a nonself competitor. Despite the lack of significant differences in producer cell densities, comparing the growth rate of the producer strain in pure and mixed cultures revealed an 11.6% reduction in producer growth in the mixed treatment at 6 hours; although, this difference was not significant ($F_{1,5} = 1.20$; $p = 0.32$). To determine whether this pattern was indicative of a difference in the competitive environment experienced by the producer cells, we ran 10 additional replicates of the producer alone and in the presence of Bov59R. Growth rates were found to be significantly lower in the mixed treatment at 6 hours (Figure 5, $F_{1,5} = 10.95$; $p = 0.021$). Producer growth rate was reduced by 11.3% in the follow-up experiment, consistent with the reduction observed in the initial experiment. The difference in growth rate did not persist to 24 hours (Figure 5, $F_{1,5} = 1.10$; $p = 0.34$).

Discussion

Bacteriocins are a class of allelopathic toxins that are ubiquitously produced across microbial lineages despite being highly costly (Riley & Wertz, 2002a; Riley & Chavan, 2006; Wloch-Salamon *et al.*, 2008). While theory predicts that allocation to bacteriocin production should be

increased when producer cells occur at intermediate frequencies relative to nonself competitor cells (Gardner *et al.*, 2004), the degree to which bacteriocin production is phenotypically plastic and induced by the presence of nonself competitors remains underexplored. We tested the plasticity hypothesis using natural isolates of *Xenorhabdus spp.*, by examining bacteriocin production at various time points over the course of growth when producer cells were grown in pure culture, or in co-culture with a nonself competitor strain. Our results show that bacteriocin production can be detected during the late exponential phase (12 hours) and the stationary phase (24 hours), regardless of the presence of nonself competitors. Thus, there is no evidence for increased bacteriocin production in the presence and absence of non-self competitors (Figure 2). While bacteriocin activity in both treatments reflected an almost 7-fold increase between 12 and 24 hours, this increase occurred without any concomitant increases in producer cell densities at these time points (Figure 3), indicating that bacteriocin production may be increasing on a per cell basis between these time points. However, bacteriocin activity detected at each time point in these experiments is cumulative and reflects the activity of bacteriocin produced over the entire course of growth until time of sampling. Therefore, the increase in bacteriocin production between 12 and 24 hours may be also explained solely by the accumulation of bacteriocin without invoking a per capita increase in toxin production. Overall, these results provide no evidence to suggest that bacteriocin production is increased in response to the presence of nonself competitors.

We set out to test the prediction that bacteriocin production increases in co-culture with nonself competitors, when the benefits of toxin production can be realized by the producer population. However, bacteriocin production may not be expected to increase in the presence of

nonself cells if the nonself cells do not impose increased competition on the producers. To determine whether the nonself cells used in our experiment imposed increased competition on producer cells, we compared the growth rate of the producer strain in the presence and absence of the nonself competitors at 6 hours and 24 hours. Results revealed that producer cells had significantly reduced growth rates in the mixed culture at 6 hours (Figure 5), indicating that the producer cells experienced increased competition due to the presence of Bov59R cells in the mixed culture early on in the experiment. Bacteriocin production was examined over the course of growth (Figure 3) encompassing time points when the presence of Bov59R imposes increased competition on producer cells in co-culture. However, our results did not provide any evidence for increased bacteriocin activity in the presence of the nonself competitor at any examined time points (Figure 3).

Bacteriocin production is costly. In addition to costs of protein synthesis and genetic maintenance, many species of gram-negative bacteria require producer cells to lyse for bacteriocin release (Riley & Wertz, 2002b; Riley & Chavan, 2006; Wloch-Salamon *et al.*, 2008). Consequently, only a small proportion of the producer population is actively engaged in bacteriocin production and release at a given time. In *E. coli*, this frequency has been estimated to range between 0.5% and 9% by using gfp-reporter strains linked to bacteriocin promoters (Mulec *et al.*, 2003; Bayramoglu *et al.*, 2017). While similar estimates for the frequency of cells actively producing bacteriocin remain to be determined for the natural isolates of *Xenorhabdus spp.*, our functional assays clearly show no evidence in support of the hypothesis that bacteriocin production increases in the presence of nonself competitors. Recently published work demonstrates that *E. coli* populations increase colicin production in response to attacking

competitors (Mavridou *et al.*, 2018). Intriguingly, the competitor strain used in our experiment does not attack the producer strain. This contrast suggests the possibility that plastic increases in toxin production may be more likely to occur when producer cells experience direct attack, and this hypothesis may be explicitly tested in future investigations.

The time course of bacteriocin production in our results suggests that bacteriocin production increases in stationary phase. These results are consistent with previous findings using *E. coli* (Eraso *et al.*, 1996). Taken together, these results suggest that toxin production may be triggered by generic cues associated with competition such as nutrient limitation, arising from increasing cell densities, instead of specific mechanisms of self/nonself detection. Such a mechanism of competition sensing via generic cues is also consistent with the 'competition sensing hypothesis' (Cornforth & Foster, 2013). The ability of bacterial cells to detect and respond to nonself genotypes has been documented in multiple species (Gibbs *et al.*, 2008; Garbeva *et al.*, 2011; Korgaonkar & Whiteley, 2011; Unterweger *et al.*, 2012; Basler *et al.*, 2013; Wenren *et al.*, 2013). However, tailoring specific responses to different nonself genotypes may be highly costly and require specialized receptors to detect specific competitor cues. Moreover, competitor cues may also evolve to evade detection. Thus, it has been suggested that sensing general cues associated with competition may be more useful than relying on self/nonself recognition (Cornforth & Foster, 2013).

An important criterion for a phenotypically plastic response to be effective is its timeliness (Padilla & Adolph, 1996). Plasticity in bacteriocin production may be unfavorable if induced bacteriocin production is too slow, thereby allowing a growth advantage to the

competing strain while the producer first detects and then responds by producing bacteriocins. The *Xenorhabdus spp.* isolates used in this study are entomopathogenic bacteria which form mutualistic associations with *Steinernema* nematodes to enter an insect host (Martens *et al.*, 2003; Stock & Blair, 2008). Quick growth is crucial for the successful colonization of the insect host as well as for the competitive success of these bacteria (Bashey *et al.*, 2011). In fact, in some cases, a sensitive but faster growing isolate can outcompete a producer strain (Bashey *et al.*, 2013) demonstrating the importance of timing in the within-host competitive environment.

Further, if producers frequently encounter sensitive, nonself competitors in their natural habitat, selection for plasticity in response to a nonself competitor may not be strong. The benefits of bacteriocin production during frequent encounters with sensitive competitors may outweigh the costs of toxin production on rare occasions when the producer does not encounter a competitor. These conditions seem plausible for the species used in this study. Numerous genotypes of *Xenorhabdus spp.* have been found at an ecologically relevant scale (Hawlena *et al.*, 2010a; b) suggesting that the presence of nonself competitors may be ubiquitous in nature, thus further reducing the selection for plasticity. Future investigations may employ experimental evolution to investigate whether bacteriocin production differentially evolves when competitors are reliably encountered in the environment or not.

In highly diverse communities with numerous producing, sensitive, and resistance genotypes, bacteriocin production may be maintained through non-transitive interactions resulting in frequency-dependent selection (Kerr *et al.*, 2002). Additionally, the prediction that bacteriocin production should be plastically increased in response to nonself competitors may not

hold up if bacteriocins serve other functions in addition to anticompteitor roles. It has been suggested that for microbes that form symbioses with other organisms, antimicrobial toxins may also serve as honest signals to facilitate mutualist pairing (Hillman & Goodrich-Blair, 2016). If bacteriocins serve as important signals to attract nematode mutualists, then production would be beneficial and necessary even in the absence of bacterial competitors. If so, once producer populations reach high densities, it may be time to find mutualist partners within the host and release bacteriocins irrespective of the presence of nonself competitors. Further investigation to examine the consequences of manipulating bacteriocin production on effective symbiont association could influence the way costs and benefits of bacteriocin production are understood and shed light on the regulation and maintenance of bacteriocins in nature. In addition to bacteriocins, allelopathic traits in other taxa may also not be plastically responsive to the presence of nonself competitors for analogous ecological reasons. Overall, these results strongly highlight the need for more studies in the future aimed at investigating the constitutive versus inducible nature of anti-competitor traits and testing alternative predictions to develop a better understanding of how costly anti-competitor traits are maintained in nature.

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Figure 1

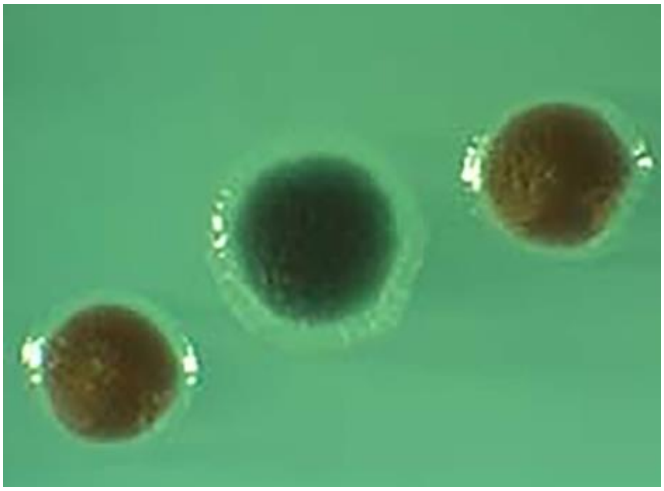

Figure 1: Image showing colonies of natural *Xenorhabdus* isolates used in the study. The producer strain *Xenorhabdus koppenhoeferi* isolate 46 (Kop 46), grows as small, maroon colonies, while colonies of the competitor strain *Xenorhabdus bovienii* isolate 59 (Bov 59) are larger and blue.

Figure 2

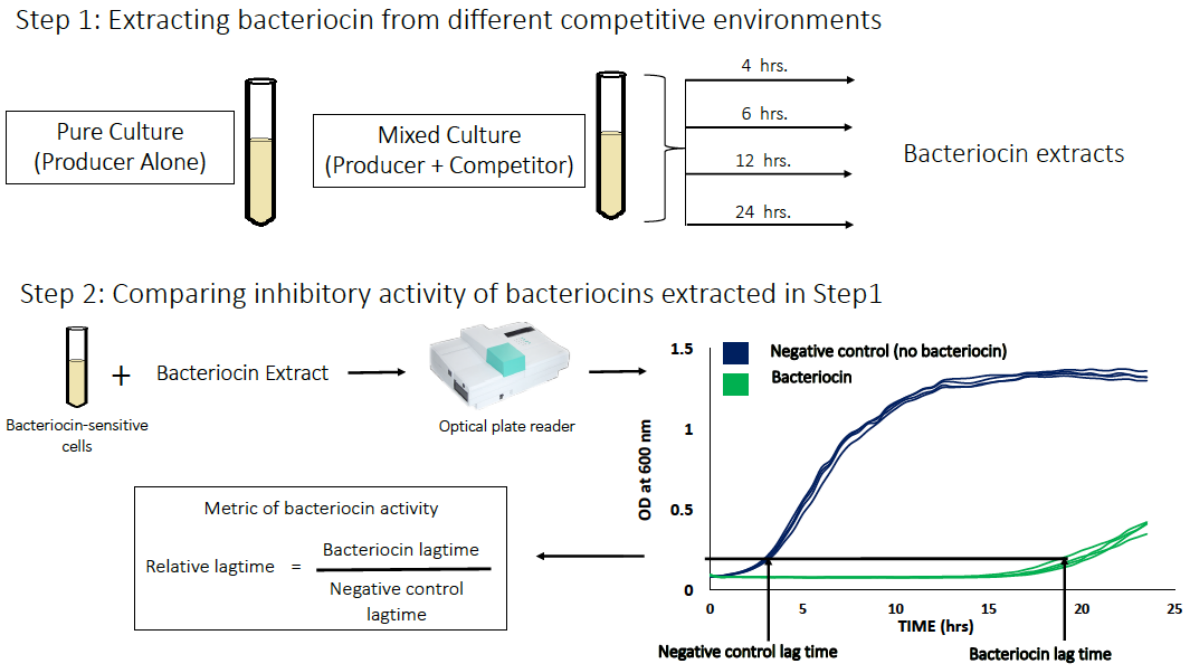

Figure 2: Schematic showing the experimental design and methods. The competitive environment of a bacteriocin producing lineage was manipulated by either growing the producer strain alone in pure culture or in the presence of a competing strain in mixed culture. Bacteriocin extracts were collected from these treatments at four different time points (4, 6, 12 and 24 hours). These extracts were subsequently tested for inhibitory activity using a growth inhibition assay. A fixed amount of a bacteriocin extract was added to a starting culture of bacteriocin sensitive cells. The growth of these cultures was tracked in an optical plate reader which measured OD_{600} readings at 30 minute intervals for 24 hours. An extract derived from a pure culture of the competitor cells which contains no bacteriocin was used as a negative control for the bacteriocin extracts. Data from the optical plate reader yields growth curves as shown. Four curves for each treatment represent curves from four replicate wells on the optical plate reader. Lagtimes were estimated for all wells and the average of four wells for each treatment was used to calculate relative lagtime as shown in figure.

Figure 3

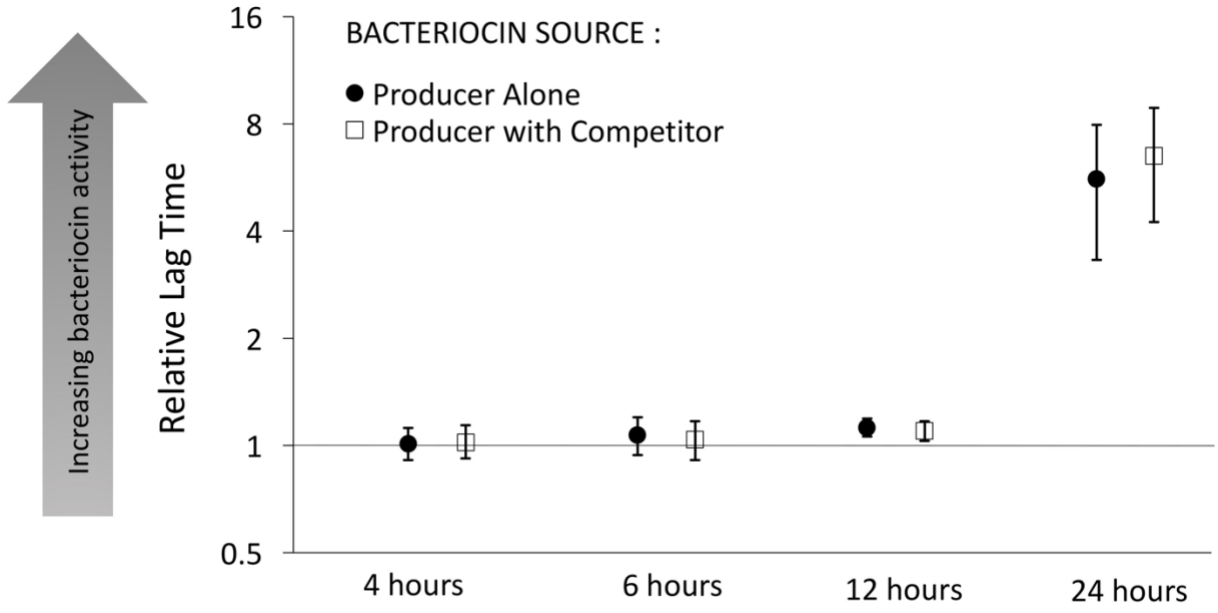

Figure 3: Relative lag times (+/- 95% confidence intervals) induced by bacteriocin extracts collected from cultures of the producer growing alone (filled-in circles), and producer growing in mixed cultures with equal starting frequencies of a competing strain (empty squares). The X-axis represent the time points at which bacteriocin extracts were collected over the course of growth. Each time point was destructively sampled. Relative lag time values greater than 1 indicate significant inhibitory activity. Relative lagtime values were calculated as shown in Figure 1.

Figure 4

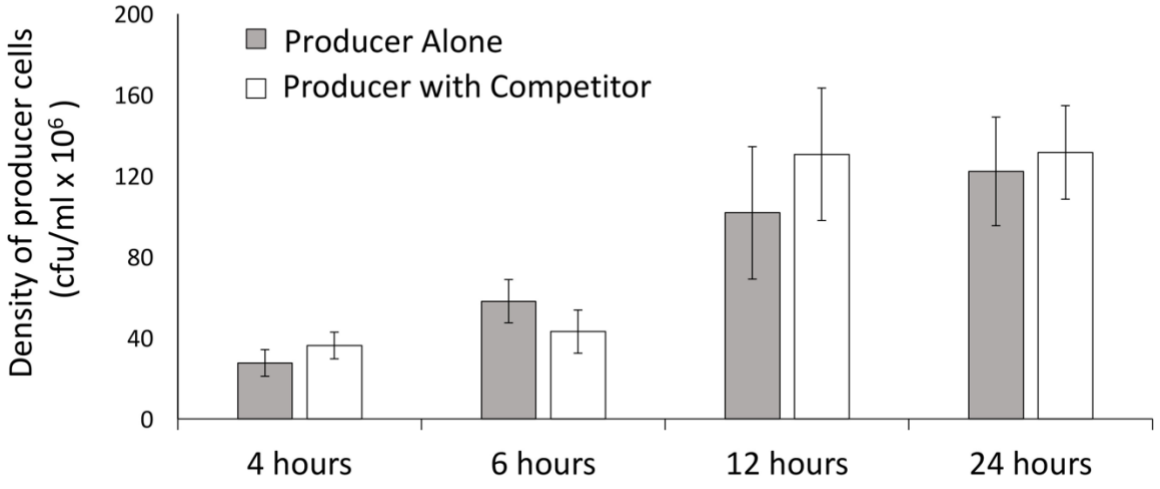

Figure 4: The density (+/- 1. S.E.M.) of cells from the bacteriocin producer strain in the pure and mixed cultures at the time of bacteriocin extractions.

Figure 5

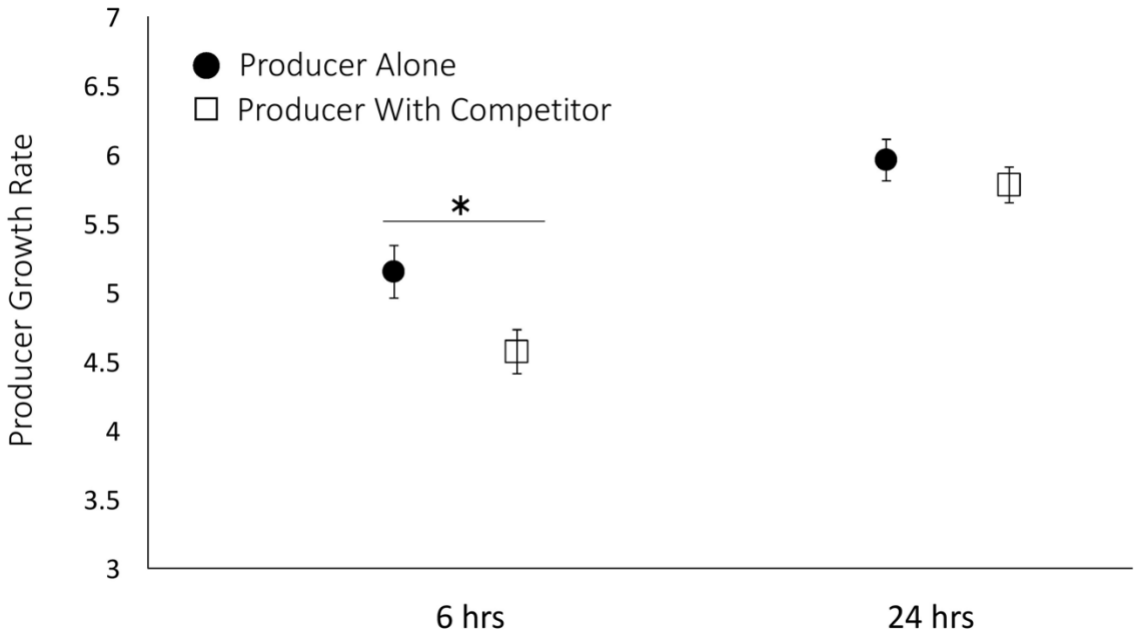

Figure 5: The growth rate of the producer strain (+/- 1. S.E.M.) in pure and mixed cultures measured at 6 and 24 hours. Growth rate at each time point was calculated as ln[final density of producer cells / initial density of producer cells]. The * indicates a significant difference between treatments ($p < 0.05$).

Supplemental Materials

S1: Choosing the competitor strain for mixed treatment – This assay was performed to determine whether the inhibitory activity of bacteriocin produced by the producer strain could be subsequently affected by the presence of the competitor strain in the mixed culture. To test whether exposure to cells can affect the inhibitory activity of bacteriocin, a fixed amount of chemically induced bacteriocin was either "Exposed" by applying it to a starting culture of cells (Bov59 and Bov59R) or "Unexposed" by adding it to the same volume of un-inoculated growth medium (Luria Broth). A pure culture of Bov59R with no added bacteriocin was used as a "No Bacteriocin" negative control. All four treatments were incubated at 28^{o}C with shaking for 24 hours. Following incubation, supernatants were collected and a growth inhibition assay was performed to compare the bacteriocin-mediated inhibitory activity across the four groups. Here, inhibitory activity is measured as the absolute lagtime (minutes) induced by the various extracts on the growth of a starting culture of bacteriocin sensitive Bov59 cells. Absolute lag times induced by the no bacteriocin negative control, unexposed bacteriocin and the bacteriocin extracts exposed to either Bov59 or Bov59R were compared using a mixed model analysis of variance with 'bacteriocin source' as fixed effect and experimental replicate as random effect.

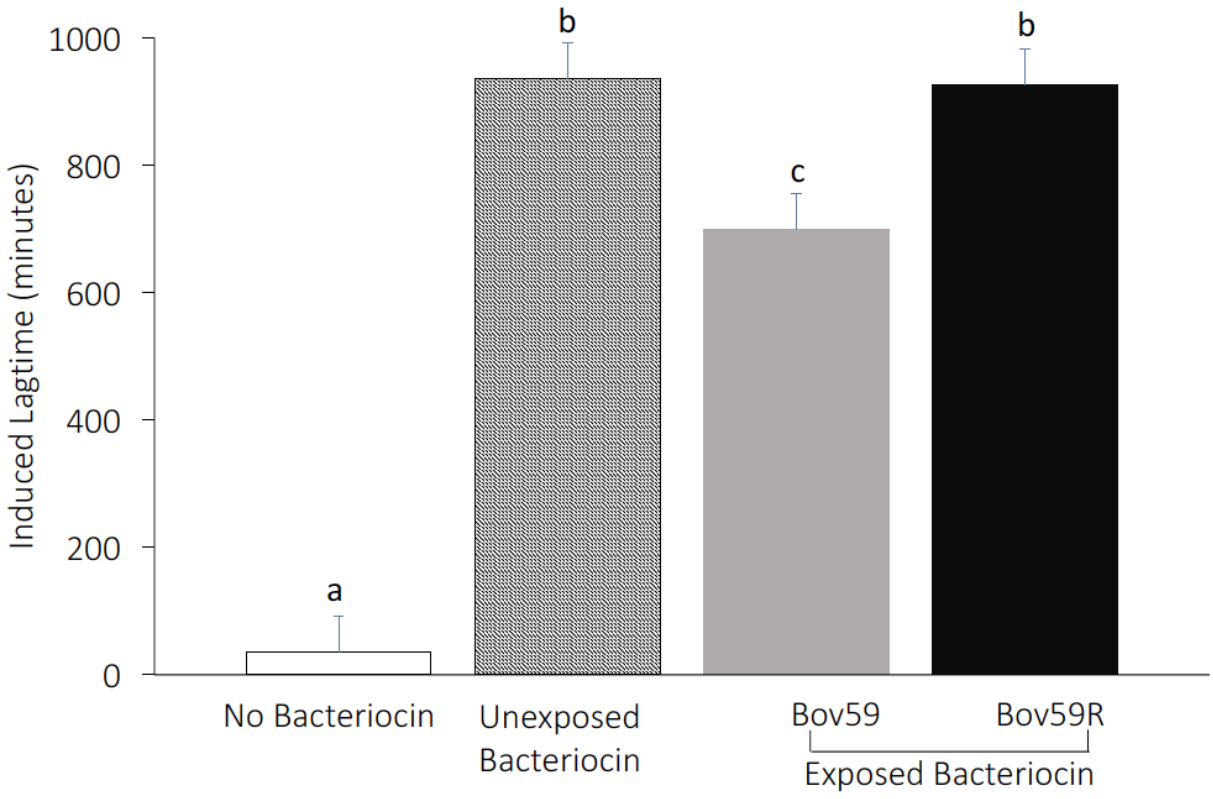

Figure S1: Inhibitory activity of bacteriocin exposed to Bov59 and Bov59R cells in comparison with bacteriocin that is unexposed to any bacterial cells. Bacteriocin that was previously exposed to Bov59 cells showed a significant reduction in inhibitory activity compared to bacteriocin which was previously unexposed to cells but otherwise similarly treated. In contrast, exposure to the lab-derived Bov59R strain showed the same inhibitory activity of bacteriocin as unexposed bacteriocin. Following these results the Bov59R strain was chosen as the competitor strain for the mixed treatment. Mean lagtime (+/- Standard error) are plotted in minutes; the letters (a,b,c) indicate statistically significant differences between treatments at $p<0.05$.

S2: Sensitivity of the growth inhibition assay – The growth inhibition assay was performed using three different starting cell densities ($10^{\wedge 6}$, $10^{\wedge 5}$ and $10^{\wedge 4}$ c.f.u/ml) of the sensitive detector culture. Three different dilutions of chemically induced bacteriocin were applied to starting cultures at each density. A no bacteriocin negative control was applied to each starting cell density as well. The difference in lagtimes induced by a bacteriocin dilution and the respective negative control was used as a metric of assay sensitivity. A greater difference in the induced lagtimes between negative control and bacteriocin samples provides an increased window for detecting bacteriocin activity. Each bacteriocin dilution- x- cell density combination was tested on four replicate wells in the optical plate reader. The induced lagtime for each well was subtracted from the mean induced lagtime of the relevant no bacteriocin control. The average differences in induced lagtimes with respective standard error of means are plotted in Figure S2.

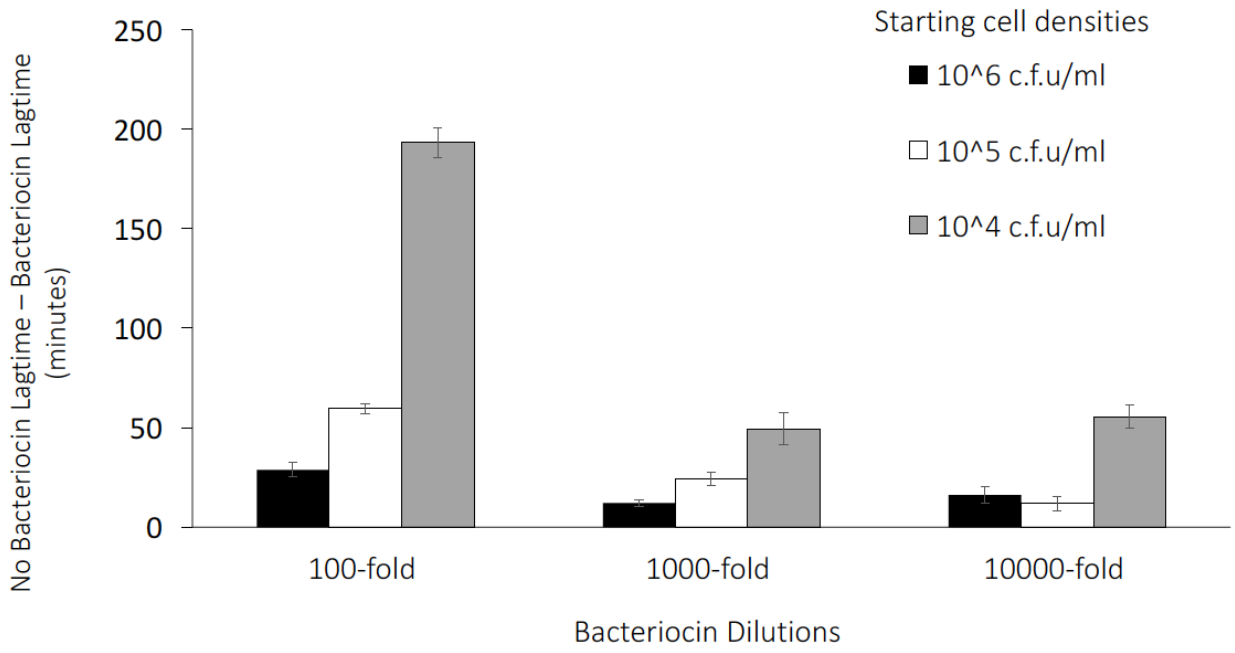

Figure S2: Sensitivity of growth inhibition assay increases with decreasing starting cell densities of detector culture. The difference between lagtime induced by a no bacteriocin

negative control and the lagtime induced by a fixed concentration of chemically induced bacteriocin increases when growth inhibition assay is performed using lower starting cell culture densities. Shown here are three different dilutions of chemically induced bacteriocin (100-fold, 1000-fold and 10000-fold) tested across three different starting cell densities. For any bacteriocin dilution, the difference in lagtimes between negative control and bacteriocin dilution is highest at the lowest starting cell dilution. This increased difference in the lagtimes makes it more likely to detect inhibitory effects of low concentrations of bacteriocins. The error bars represent standard error of mean.

CHAPTER 2

The evolution of spiteful bacteriocin production

A. Bhattacharya and C. M. Lively

Abstract

A premier example of spite in nature is the bacterial production of anti-competitor toxins, called bacteriocins, which can kill closely related competitors. The synthesis of these toxins is considered spiteful due to their high costs of production. In addition, the release of bacteriocins is often considered a 'suicide mission,' as many species require cell lysis to expel their toxins. Despite such costs, bacteriocin production is ubiquitous in bacteria. How did such a costly strategy become so common? Here, we used a modeling approach to investigate the invasion of a rare, toxin-producing mutant in a population dominated by a faster-growing sensitive strain. First, we examined local competition between a bacteriocin-producing mutant and a bacteriocin-sensitive strain within a focal resource patch. Our results showed that bacteriocin release upon natural cell death can be sufficient for the bacteriocin producer to outcompete a faster growing sensitive competitor within the focal patch. Next, we examined whether the bacteriocin-producing mutant could invade the larger metapopulation composed of non-focal resource patches colonized by the sensitive strain. We found that bacteriocin production can invade a metapopulation when the scale of competition is partially local. Further, we found that plastic bacteriocin release via inducible cell deaths (suicides) in response to the presence of sensitive competitors can promote, but is not necessary, for the invasion of the producer strain. We discuss

the relevance of these results for natural populations, and present a specific example of canalized spite, which can be explained by the model.

Introduction

The evolution and maintenance of spiteful behaviors that reduce the fitness of both the actor and the recipient pose a long-standing evolutionary puzzle (Hamilton, 1964, 1970). How can a trait that reduces the fitness of the actors and the recipients be favored by natural selection? One of the clearest examples of spite in nature is the production of anti-competitor toxins by bacteria, known as bacteriocins (Riley & Chavan, 2006; West *et al.*, 2007a). Bacteriocins are proteinaceous toxins with diverse mechanisms of action, which can kill closely related local competitors without killing clone mates (Reeves, 1965; Riley & Chavan, 2006). However, the costs of toxin synthesis often result in slower growth of the producing strains, rendering the trait spiteful (West *et al.*, 2007). In addition, the release of these large, proteinaceous toxins often requires cell lysis, particularly in gram-negative cells, which lack a cell wall (Riley and Wertz 2002; Riley and Chavan 2006). As such, discussions of bacteriocin production in the literature often assume that bacteriocin release is a product of additional cell death ('suicide') for the sole purpose of bacteriocin release, thus imposing extremely high direct costs of production on individual producer cells (e.g., Nedelcu *et al.*, 2010; Mitri & Foster, 2013). Despite being costly, bacteriocin production is ubiquitous in bacteria (Klaenhammer, 1988; James *et al.*, 1991).

Previous work suggests that the competitive environment maybe an important factor in the evolution of bacteriocin production (Chao & Levin, 1981; Gardner *et al.*, 2004). Using a game theoretical approach, Gardner *et al.* (2004) showed that the optimal level of resource allocation to bacteriocin production varies according to relatedness in a competitive arena. Their

model predicts that relatively high investment in bacteriocin production is favored under intermediate relatedness. Additionally, models suggest that spite is more likely to be favored under local competition than global competition (Gardner & West, 2004; Gardner *et al.*, 2004). Consistent with these predictions, experimental work has shown that the relative fitness of a bacteriocin-producing *Pseudomonas aeruginosa* lineage is highest relative to a non-producing mutant at intermediate relatedness in local competition with a sensitive competitor (Inglis *et al.*, 2009, 2011).

The theory and experiments discussed above suggest that investment in bacteriocin production is most favored when producers and competitors occur at comparable frequencies (Gardner *et al.*, 2004; Inglis *et al.*, 2009). As such, it is reasonable to hypothesize that phenotypically plastic bacteriocin production – in which bacteriocins are released when competitors are present – may be favored by selection. A recent meta-analysis led to the proposition of the 'competition sensing hypothesis' which posits that bacterial lineages may have evolved to preferentially respond to competition-associated stressors such as cellular damage and starvation, by triggering bacteriocin production (Cornforth & Foster, 2013). However, there have been few direct empirical tests of plasticity in bacteriocin production and their results remain equivocal. Bacteriocin release following 'provocation' or toxin-mediated killing has been documented in *E. coli* strains (Majeed *et al.*, 2011, 2013; Gonzalez *et al.*, 2018). Similarly, a recent study showed that *E. coli* producer colonies increased production of bacteriocins when neighboring colonies of competitors invaded spatially structured environments (Mavridou *et al.*, 2018). In contrast, a study of the insect-pathogenic bacteria, *Xenorhabdus spp*. found no evidence for increased bacteriocin production in the presence of non-self competitors

(Bhattacharya *et al.*, 2018). *In vitro* studies in *E. coli* as well as *Xenorhabdus spp*. have detected bacteriocin production in the absence of competitors during late growth stages of laboratory batch cultures (Eraso *et al.*, 1996; Bhattacharya *et al.*, 2018). This may be consistent with the 'competition sensing hypothesis' (Cornforth & Foster, 2013), whereby starvation caused by resource depletion triggers bacteriocin production. But if cell lysis is required for bacteriocin release, it is puzzling to imagine why cells would commit 'suicide' in the absence of competitors. In the current study we explicitly investigate whether such plasticity is necessary for the invasion of bacteriocin production and examine the conditions that could favor the evolution of plasticity in bacteriocin production.

Here we use a modeling approach to examine the simplest conditions for a rare, bacteriocin-producing mutant to invade a metapopulation composed of numerous resource patches colonized by a faster-growing, sensitive strain. To simplify the model, we assume that each new patch is colonized by a fixed, initial number of bacterial cells. In the metapopulation, every resource patch is colonized by the sensitive strain except one patch which we the 'focal patch'. In the 'focal' patch half of the colonizing cells belong to a bacteriocin-producing mutant, and the other half belong to the resident, sensitive strain. We first investigate the conditions that would be necessary for the producing lineage to 'win' against the sensitive competitor in this focal patch. To do this, we develop a resource-based model that incorporates explicit demographic details about population growth, and bacteriocin potency. Importantly, we assume that bacteriocin production imposes a cost on producer births, and requires cell lysis for release. We then investigate the conditions necessary for this bacteriocin-producing strain to invade the metapopulation when there are different levels of competition within vs. between patches. Our

results show that bacteriocin release by lysis upon natural cell death is sufficient for bacteriocin producers to spread. We also examine competitor-induced plasticity in bacteriocin release; we find that plastic bacteriocin release can promote the invasion of bacteriocin producers under some conditions, but plasticity is not necessary for the early evolution and spread of bacteriocin production.

Model

Imagine a metapopulation comprising a large number of resource patches. Each new patch contains a fixed amount of resources and is initially colonized by a fixed number of bacterial cells. Every colonized patch except one is colonized by a resident strain that does not produce bacteriocins. The remaining colonized patch, which we call the 'focal' patch (following Frank, 1998), is colonized by a bacteriocin-producing mutant and the resident strain in equal frequencies. The bacteriocin produced by the mutant strain can kill cells of the resident, sensitive strain. However, the sensitive strains grow faster because bacteriocin-producing mutant pays a growth cost of bacteriocin production. We examine the simplest conditions that would be necessary for this bacteriocin producing strain to (a) outcompete the sensitive strain within the focal patch and (b) invade and spread through the metapopulation under varying levels of within vs. between patch competition.

Our model differs from previous formulations used to investigate the maintenance of bacteriocin production in a few ways. Previous models have derived the optimal investment in bacteriocin production (Gardner *et al.*, 2004), assuming competition between two or more producing strains of bacteria (Gardner *et al.*, 2004; Majeed *et al.*, 2011), whereas we focus on the

fate of a rare mutation that causes bacteriocin production in a population of sensitive individuals that do not produce toxins (but see also Durrett & Levin, 1997). Our model also defines the resource environment and assumes that every resource patch has a limited amount of resources. Finally, our model includes explicit demographic details like values for birth and death rates of the competing strains, allowing us to examine mechanisms that could not be addressed by previous models. Specifically, we examine: (a) whether bacteriocin-release upon natural cell deaths is sufficient for the invasion of bacteriocin production , (b) whether phenotypically plastic 'suicides' in the presence of competitors can affect the evolution of bacteriocin production, and (c) how the timing of dispersal with respect to resource availability affects invasion of bacteriocin production. Note that for the case of pathogenic bacteria, dispersal to a new patch can be viewed as transmission to a new host.

Within-Patch Competition

We began by modeling resource competition between the bacteriocin producer, *P*, and a sensitive resident, *S*, within the focal patch. The producer strain pays a growth cost of bacteriocin production and bacteriocin release required cell death. However, no additional cell death was invoked for the specific purpose of bacteriocin release. Our model made the following assumptions:

Producer and Sensitive lineages have equal founder population sizes in the focal patch

The intrinsic death rate, *d*, is equal for both lineages

The birth rate, *B*, and death rate, *D*, are both density-dependent

Producer births is equal to Sensitive births minus the cost of bacteriocin production

Sensitive deaths is equal to Intrinsic deaths plus deaths caused by bacteriocins

Bacteriocins require cell lysis for release

Table 1 describes the terms used in the model.

The amount of available resources, *R*, was fixed and not replenished. We chose this resource structure to represent common, ephemeral habitats in nature. All natural cell deaths occurred only after resources were exhausted. Density-dependent birth (B_t) and death (D_t) rates, the total numbers of producer (P_t) and sensitive cells (S_t), and the number of resource units (R_t) were computed at every time step, *t*, for 'T' time steps. We assumed that the birth of a new cell required one resource unit. Intrinsic birth rate, *b*, was fixed at 1 which means that the population could double its size at every time step if no deaths occurred.

The birth rate, B_t, was density-dependent and declined with increasing density. B_t was calculated as:

$$B_t = b\left[1 - \frac{N_t}{N_t+R_t}\right] \quad \text{(Eqn 1)}$$

where '*b*' is the intrinsic birth rate, and N_t is the total number of cells ($N_t = P_t + S_t$).

Similarly, the death rate, D_t, for natural cell deaths was also density-dependent and calculated as:

$$D_t = d\left[\frac{N_t}{N_t+R_t}\right] \quad \text{(Eqn 2)}$$

where *d* is the intrinsic death rate. As described below, this death rate was applied to population growth equations only after resources were depleted. The model kept track of the amount of resources left in the system and D_t was set to 0 until resources were depleted.

The number of producer cells entering the next time step was calculated as:

$$P_{t+1} = P_t[1 + B_t(1 - x) - D_t] \quad \text{(Eqn 3)}$$

where *x* is the selection coefficient against represents the birth-rate cost of bacteriocin production. Similarly, the number of sensitive cells entering the next time step was calculated as:

$$S_{t+1} = S_t[1 + B_t - D_t - D_t P_t S_{killed}] \quad \text{(Eqn 4)}$$

where S_{killed} was the number of sensitive cells killed by bacteriocins released from the death of one producer cell.

At every time step, the amount of resources remaining in the system were calculated by deducting the number of new producer and sensitive births in that time step from the resources available prior to those births:

$$R_{t+1} = R_t - P_t B_t(1 - x) - S_t B_t \quad \text{(Eqn 5)}$$

When the number of resource units remaining in the system was insufficient for any more births to occur ($R_t < 1$), 'StationaryPhase' began. The 'stationary phase' was included to mimic the growth phase commonly observed during *in vitro* bacterial growth in cultures when there are no new births or deaths. As this point the *counter* variable began counting time steps for stationary phase. In the present model, when the *counter* reached a value of 6, natural deaths begin to occur. The model used conditional 'if-then' statements to set the death rate D_t as 0 in the equations before *counter* reached the chosen value and applied Eqn 2 to calculate D_t after *counter* reached the chosen number of generations. Note that all results were qualitatively robust to the existence and length of 'StationaryPhase'.

Invasion of the metapopulation

The within-patch model examines the invasion of a rare, bacteriocin-producing mutant which was present only in the 'focal patch'. For bacteriocin production to succeed as a strategy, this rare strain must spread through the entire metapopulation of faster-growing, sensitive strains. The likelihood of the producer strain invading the metapopulation may depend on the relative importance of within-patch versus between-patch competition, also known as the 'scale of competition' (Frank, 1998 (pp. 114–133); Gardner & West, 2004; Lively, 2009). We calculated the relative fitness of the bacteriocin-producing strain across various scales of competition, a, ranging from strictly local competition (a =1) to strictly global competition (a = 0). Strictly local competition (a = 1) can be imagined as a scenario in which there is only a single resource patch. On the other hand, global competition (a = 0) represents a scenario where the producer strain's relative fitness is determined by its production of daughter cells relative to average production of daughter cells by sensitive strains in the non-focal patches. Intermediate values of the scale of competition describe the relative importance of local vs global competition. In nature, the scale of competition may be determined by various mechanisms including spatial structure, viscosity, or mechanisms of dispersal. Regardless of the specific mechanism, we investigated how the scale of competition affects whether bacteriocin-production, as a strategy, can invade a metapopulation of non-producing, sensitive strains.

The relative fitness of bacteriocin producers (w_{prod}) was calculated as (following Frank, 1998):

$$w_{prod} = \frac{W_{prod}}{a\,\bar{W}_{local} + (1-a)\bar{W}_{global}} \qquad \text{(Eqn 6)}$$

where, W_{prod} is the number of producer cells (P_t) at dispersal in the focal patch; $\bar{W}_{local}$ is the average number of cells per strain in the focal patch at dispersal, i.e., (P_t + S_t)/2; and $\bar{W}_{global}$ is the average number of cells per strain in the non-focal patches at dispersal, i.e., S_{tot}/2, where S_{tot} is the total number of sensitive bacteria in an average non-focal patch at dispersal. Note that for consistency across patches we assume there are two 'sensitive' strains in the non-focal patches (hence, S_{tot}/2), just like there are a total of two strains (one producer and one sensitive) in the focal patch. This is also reflected in the total number of bacterial cells that are present in the focal and non-focal patches. The non-focal patches have twice as many sensitive cells to begin with as the focal patch.

The relative fitness of the producer strain, w_{prod}, was examined at two different time points representing dispersal: one before resources are exhausted (Generation 20) and another after resources have been exhausted (Generation 40).

Competitor-induced plasticity in bacteriocin release

Finally, we examined whether phenotypically plastic bacteriocin release by the producers would be favored by selection. Since bacteriocin release requires cell lysis and death, plastic bacteriocin release would result in additional cell deaths beyond natural cell deaths. Thus, to examine competitor-induced plasticity in bacteriocin release, the within-patch competition model was modified to include additional death rate, *z*. Specifically, the additional producer deaths due to plasticity was calculated as

$$\frac{zS_t}{N_t+R_t} \qquad \text{(Eqn 7).}$$

Note that according to this formulation, plastic producer-cell deaths depend on both the frequency of the sensitive cells as well as the availability of resources. Both of those factors have

been implicated in predictions about competition sensing (Cornforth & Foster, 2013). However, qualitative results in the model are robust to formulations that examine only sensitive strain frequency, or only total density.

Plastic deaths were incorporated from the beginning of growth until 'stationary phase' ended. After stationary phase ends, all deaths are calculated as natural cell deaths, according to the intrinsic death rate. This was incorporated to the model by modifying the Eqn 3 to include an additional death term for plastic deaths as follows:

$$P_{t+1} = P_t \left[1 + B_t(1 - x) - D_t - \frac{zS_t}{N_t + R_t}\right] \quad \text{(Eqn 8)}$$

When $z = 0$, or if sensitive cells, S, are eliminated from the population, the producer does not incur any inducible cell deaths. Invasion of the metapopulation was evaluated as described above.

The case of frequency-dependent dispersal

Here we use the model to examine the invasion dynamics of the bacteriocin-producing lineage when dispersal is frequency dependent. To do so, we imposed a limit on the total number of bacterial cells that can disperse from any given resource patch. For simplicity we set this limit to the initial number of cells that colonized new resource patches. The number of producer cells that disperse from the focal patch and contribute to the producer's relative fitness was determined by the producer strain's relative frequency within the focal patch at dispersal. The

number of sensitive cells that dispersed from the non-focal patches were determined by the imposed limit as the frequency of sensitive strains in the non-focal patches was always 1.

Results

Within-Patch Competition

We found that a bacteriocin-producing strain cannot outcompete sensitive cells if the bacteriocin does not kill any competitors ($S_{killed} = 0$), reflecting the cost of bacteriocin production (Figures 1a,b). However, when bacteriocin production results in a 5% reduction in fitness ($x = 0.05$), an average value of $S_{killed} = 0.5$ was sufficient for the producing lineage to outcompete the sensitive lineage (Figures 1 c, e, g). At $x = 0.1$, the producer population was able to outcompete sensitive cells at S_{killed} values of 1 or more (Figures 1 f, h). Greater bacteriocin potency (S_{killed}) allowed producers to overtake sensitive cells faster. Across both levels of costs (x), as the potency of the bacteriocin (S_{killed}) increased, the number of time steps required by the producers to outcompete the sensitive cells decreased. It is important to note that in all cases where the producer 'won', it was only able to do so after resources were depleted and natural cell deaths began.

Invasion of the metapopulation

The results of the within-patch competition showed that a bacteriocin-producing strain could outcompete a faster-growing sensitive competitor after resources were depleted. Next we examined if the producer strain could invade a metapopulation of sensitive competitors. Here, we fixed the cost of bacteriocin production at $x = 0.1$ and potency of bacteriocin production, S_{killed}, at

10 (Figure 1h) . We examined whether the producer could invade when dispersal occurred before (Generation 20) and after (Generation 40) resources were depleted (Figure 2a). This specific case (Figure 1h) was chosen for simplicity but the analysis could be performed at any fixed values of x and S_{killed} (Figure 1 c-g) by choosing specific dispersal times before and after resources are depleted under those conditions. Our results showed that the bacteriocin producer could invade the metapopulation of sensitive competitors if dispersal occurred after resources were depleted (Figure 2b). However, some degree of local competition was necessary for producers to invade; producers failed to invade when the scale of competition was less than 0.3 (Figure 2b).

Competitor-induced plasticity in bacteriocin release

We examined whether and how incorporating phenotypic plasticity in the release of bacteriocins affected the competitive outcome within the focal patch and the invasion of producers through the metapopulations. Inducible cell deaths are determined by the plastic death rate parameter, z, as well as the density and frequency of sensitive competitors (Eqn 2).

Results showed that increasing inducible cell deaths allowed the producer strain to outcompete the sensitive strain faster during within-patch competition (Figures 3 b,c). Incorporating inducible deaths also promoted the invasion of the producer strain by allowing the producer strain to invade across a greater range of scale of competition values including highly global scales of competition where the producers couldn't invade without plasticity (Figure 3 e,f). However, the increase in Wprod due to plasticity decreased with increasing local competition indicating that plasticity becomes less beneficial at increasingly local scales of competition. Plastic bacteriocin release allowed producers to invade when dispersal occurred

before resources were depleted (Figure 3 e,f ; Generation 20), which wasn't possible without plasticity (Figure 2 d-f; Generation 20).

The case of frequency-dependent dispersal

We examined how the invasion of the bacteriocin-producing lineage could be affected if dispersal was assumed to be frequency dependent instead of density dependent. Results show that under frequency-dependent dispersal, the producer population can invade across all scales of competition without plasticity (Figure 4). Further, the relative fitness of the producer is at its maximum possible value across all scales of competition, eliminating the possibility for plasticity to benefit the invasion of producers.

Discussion

Almost all known lineages of bacteria exhibit a spiteful trait, bacteriocin production (Klaenhammer, 1988; James *et al.*, 1991; Riley & Wertz, 2002). Bacteriocins are proteinaceous anti-competitor toxins that can kill closely related strains without killing clone mates, but incur growth costs of bacteriocin production rendering the trait spiteful (Riley & Chavan, 2006; West *et al.*, 2007a). Our goal here was to mechanistically examine how such a costly trait could spread when first introduced into a population of faster-growing sensitive cells. To investigate this question, we used an invasion analysis in a metapopulation framework. First, we examined competition between a bacteriocin-producer and a sensitive strain within a single resource patch. Next, we examined the conditions required for a rare producer strain to invade and spread through a metapopulation of sensitive strains. Finally, we asked if and how phenotypically plastic bacteriocin release via inducible cell deaths could affect the invasion and spread of

bacteriocin production in the metapopulation. Our results suggest that bacteriocin release upon natural cell death can be sufficient to outcompete sensitive competitors within a patch and invade a metapopulation of sensitive strains. Producer invasion depended on the timing of dispersal and scale of competition. Competition-induced cell deaths for bacteriocin release extend the conditions under which producers can invade but such plasticity is not necessary for successful invasion and spread of bacteriocin producers through the metapopulation.

It is important to note that the model presented here makes conservative assumptions about the costs of bacteriocin production and the potency of bacteriocins. The costs of bacteriocin production assumed in the model impose a 5% or 10% reduction in births, which is fairly steep. It has been previously argued that the fitness costs of bacteriocin production may be much less steep than commonly assumed in models (Dykes & Hastings, 1997). Our model examines values of bacteriocin potency as low as 0.5, which implies that the death of every producer cell results in the bacteriocin-mediated killing of 0.5 sensitive cells, on average. These values are highly conservative in the light of empirical estimates in *E.coli*, which show that every lysing producer cell is capable of releasing bacteriocin particles on the order of thousands of particles (Gordon & Riley, 1999).

Many bacteria, particularly gram-negative strains, require cell lysis and death for the release of bacteriocins (Riley and Wertz 2002; Riley and Chavan 2006). As such, many verbal models and discussions often deem bacteriocin production a 'suicide mission' whereby producer cells incur additional cell deaths for bacteriocin release (eg: West *et al.*, 2007; Nedelcu *et al.*, 2010). However, whether such 'suicides' are necessary for bacteriocin production to be maintained is not well understood. We specifically addressed this in our model. Our model

showed that bacteriocin release by natural cell death may suffice to confer a competitive advantage to the bacteriocin producer within a patch, and allowed the strain to invade a metapopulation of sensitive competitors (Figure 2). However, the timing of dispersal and the scale of competition were important determinants of the producer strain's invasion success. We examined the invasion success of the producer strain at two distinct time points: one before and one after the resources are exhausted (Figure 2a). Results suggested that the producer strain could invade if dispersal occurred after resources were depleted, but not before (Figure 2b). Our model doesn't explicitly track whether the producer genotype spreads to fixation when it can invade the metapopulation. However, the outcome of within-patch competition suggests that a rare, sensitive mutant would be unable to invade a metapopulation of producers under the same conditions, implying that an invading producer strain would spread to fixation over time. Consistent with previous work on the scale of competition (Durrett & Levin, 1997; Gardner & West, 2004; Gardner *et al.*, 2004), our results show that increasing local competition can favor the invasion of bacteriocin production (Figure 2b). Overall, the analyses of within-patch competition as well as producer invasion suggest that 'suicides' may not be necessary for bacteriocin production to be maintained.

Despite not being necessary, would phenotypically plastic, inducible cell deaths for bacteriocin release benefit the producer strain? We examined this by incorporating additional cell deaths that were determined by a plastic death rate parameter, z, that depended on the density as well as frequency of the competitor strains (Eqn 8). The formulation of these inducible deaths was also inversely proportional to the total resources available, which would be consistent with the 'competition sensing hypothesis', which posits that bacteriocin production is likely to be

induced by starvation stress (Cornforth & Foster, 2013). Our results showed that phenotypically plastic, inducible cell deaths in the presence of competitors could benefit the producer strain by allowing it to eliminate sensitive competitors faster within the focal patch (Figure 3 a-c). Faster elimination of the sensitive strain allowed the producer to invade the metapopulation even if competition was strictly global, or dispersal occurred before resources were depleted (Figure 3f). However, the benefits of plasticity depended on scale of competition. The advantage of plasticity became less dramatic as the scale of competition became more local. Regardless of inducibility, the producer strain achieved maximum relative fitness under strictly local competition (see Generation 40, Figure 3 d-f). These results indicate that biological details that can alter the scale of competition such as spatial structure or viscosity may affect whether plasticity in bacteriocin production will be favored or not. However, plastic bacteriocin release is not necessary for the invasion and spread of bacteriocin producers.

The current model can be applied to many bacterial systems that grow on resource-limited habitats and require cell lysis for bacteriocin release, especially bacterial pathogens. Here, we describe a specific example of a bacterial pathogen that has also been a key system for the study of bacteriocin production in natural populations, *Xenorhabdus spp.*(Hawlena *et al.*, 2010, 2012; Bashey *et al.*, 2012). *Xenorhabdus spp.* form mutualistic associations with *Steinernema* nematodes to gain entry into their insect hosts (Martens *et al.*, 2003; Herbert & Goodrich-Blair, 2007; Stock & Blair, 2008). Within the insect, the nematode and bacteria separate, reproduce, and kill the insect host (Burman, 1982; Dunphy & Webster, 1988). Bacteriocin-mediated interactions between co-infecting *Xenorhabdus* strains are known to occur within the insect-host (Bashey *et al.*, 2012, 2013). Cell lysis is known to be required for the

release of bacteriocins in *Xenorhabdus* (Thaler *et al.*, 1995). When host resources are depleted, the bacteria must re-associate with their nematode vectors for transmission to a new host (Burman 1982, Dunphy and Webster 1988) and just a few bacterial cells are sufficient to pair with a single nematode host (Martens *et al.*, 2003) Thus, dispersal only occurs after resources have been depleted akin to the conditions explored in our model results. However, nematode-mediated transmission between hosts is likely to impose frequency-dependent selection on the bacterial pathogens inside the host carcass.

We used our model to examine how transmission by nematode vectors would affect the invasion of *Xenorhabdus* bacteriocin producers. As mentioned before, each nematode host associates with only a few bacterial cells for transmission (Martens *et al.*, 2003). As such, regardless of the total density of bacteria, the transmission of bacterial strains to new hosts depends on their relative frequencies within the host. In order to capture nematode-limited dispersal of bacteria from the hosts (i.e., resource patches) in our model, we imposed a limit on the total number of bacterial cells that could disperse from each resource patch and the transmission of producers and sensitive from the focal patch was thus frequency dependent.. For simplicity and consistency, we set this limit on number of cells that can disperse to the initial number of cells that colonized a new resource patch in the model. Examining the invasion of bacteriocin producers under these conditions showed that any differences in the scale of competition were effectively lost when dispersal was frequency dependent, rather than density dependent (Figure 4). This result stems from the fact that between-group competition is eliminated when there is a limit on the number of dispersing bacteria. Under such circumstances, there may be no benefit of plasticity if dispersal occurs after resources are depleted. This is

because under strictly local competition ($a = 1$) and late dispersal, the producer achieved its maximum relative fitness with or without inducible deaths (see Figure 3 d-f; Generation 40). As such, our model predicts that for a system like *Xenorhabdus* spp, bacteriocin release upon natural cell deaths after resources are depleted should be sufficient for bacteriocin production to invade. Consistent with these model results, a recent study found that bacteriocins were only detectable in liquid cultures of *Xenorhabdus kopenhoefferi* during and after the stationary phase when resources had run out (Bhattacharya *et al.*, 2018). Further, the presence of a non-self competitor did not alter the amount of bacteriocin detected, suggesting the lack of non-self competitor induced bacteriocin release. Although the example discussed here is that of a specific symbiont-pathogen, it is likely that the general assumptions of our model can be applied to other virulent bacterial pathogens as well, where transmission occurs in a frequency-dependent manner after host resources are depleted.

To summarize, the model suggests that bacteriocin release by natural cell death may be sufficient to confer a competitive advantage and allow a rare bacteriocin-producing lineage to invade and spread through a metapopulation of sensitive non-producing lineages. Invoking plastic bacteriocin release in the presence of competitors may promote the invasion and spread of bacteriocin producers, but is not necessary for the spread of bacteriocin production. Relevant details about life history, dispersal, and scale of competition can influence specific outcomes and the model described here can be used for making and testing specific predictions across a wide range of bacterial systems.

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Table 1

Term	Description	Value
T	Total time steps in addition to time step 0^{th}	100
t	Current time step (range: 1 to T)	varies
b	Intrinsic birth rate of producer and sensitive populations	1
d	Intrinsic death rate of producer and sensitive populations	0.1
x	Selection coefficient for the cost of bacteriocin production	varies
S_{killed}	Number of sensitives killed by bacteriocins released from each producer death	varies
P_t	The number of producer cells at time step i	$P_0 = 100$
S_t	The number of sensitive cells at time step i	$S_0 = 100$
N_t	$Nt = Pt + St$; total number of cells	
R_t	Resource units at time step 't' Production of a cell requires one unit	$R_0 = 10^{\wedge 9}$
Counter *(Fixed Resource Model only)*	This variable keeps track of the number of time steps after $R < 1$	Initiates at 0
StationaryPhase *(Fixed Resource Model only)*	Number of time steps after $R < 1$ until mortality begins	5
$TotProdDeaths_t$	Total producer cell deaths during time step t	Initiates at 0

$TotSenDeaths_t$	Total sensitive cell deaths during time step t	Initiates at 0
$TotDeaths_t$	$TotProdDeaths_t + TotSenDeaths_t$	Initiates at 0
D_t	Death rate	
B_t	Birth rate	
$TotResInSystem_t$	Total resources in the system; $N_t + R_t$	
z	Intrinsic death rate for plastic, inducible cell deaths	varies

Figure 1

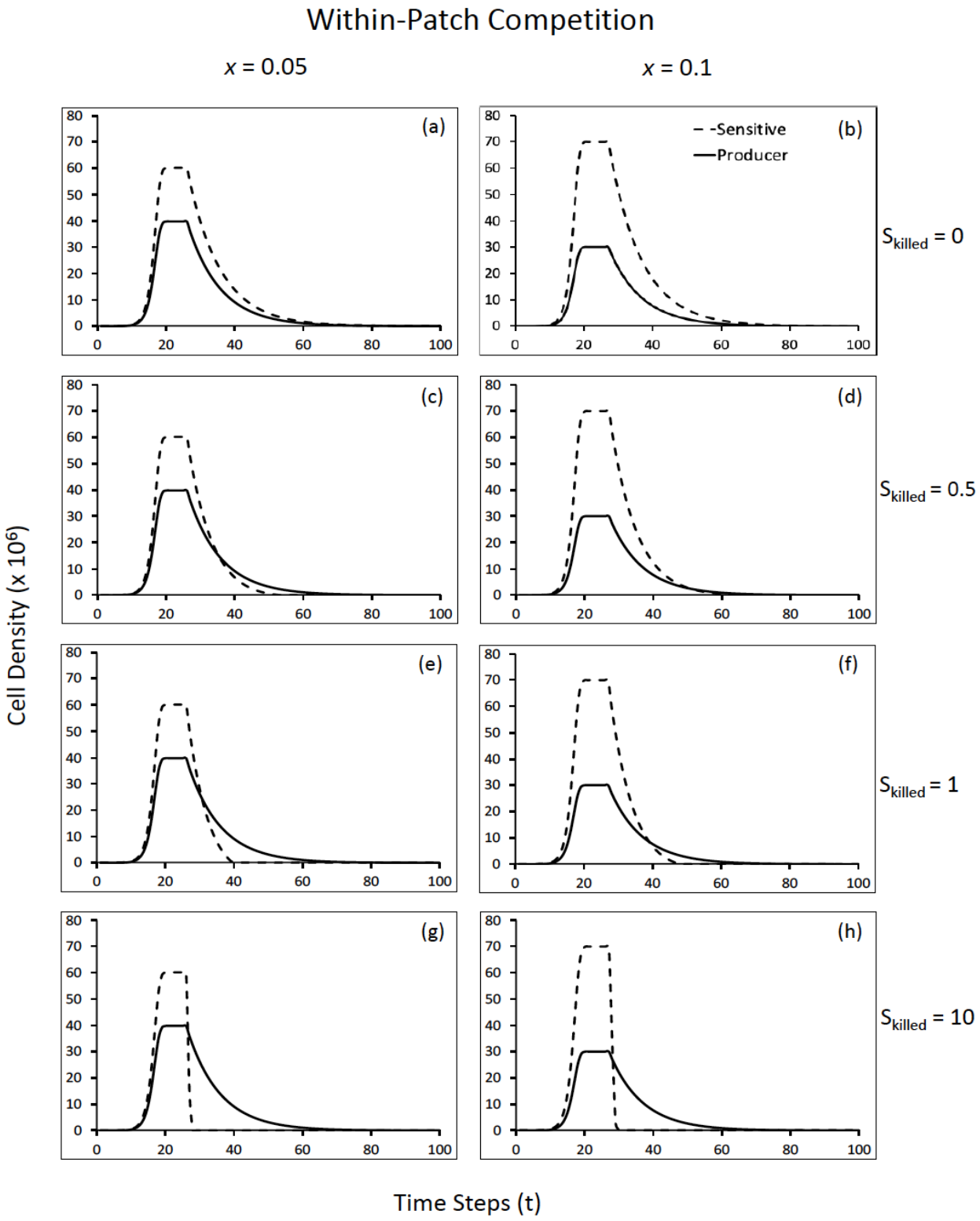
Within-Patch Competition
x = 0.05
x = 0.1
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
-Sensitive
Producer
S_killed = 0
S_killed = 0.5
S_killed = 1
S_killed = 10
Cell Density (x 10^6)
Time Steps (t)

Figure 1: Model results for 'Within-Patch Competition' showing the growth of a bacteriocin-producing lineage (Producer, solid lines) and a bacteriocin-sensitive, competitor lineage (Sensitive, dashed lines) across varying levels of cost of bacteriocin production (*x)*, and bacteriocin potency (S_{killed}). The model assumes that resources are finite and non-replenishable, and that all cell death only occurs after stationary phase when resources are depleted. The sensitive lineage can outcompete the producer lineage that pays a cost of bacteriocin production if $S_{killed} = 0$ *i.e.* when the bacteriocin cannot kill any cells (a, b). At S_{killed} values as low as 0.5 (c, d), *i.e.* when every producer cell death results in the death of 0.5 sensitive cells on average, the producer lineage can overtake the sensitive lineage (c, d). The time taken for the producer lineage to outcompete the sensitive lineage after stationary phase decreases as the potency of bacteriocin (S_{killed}) increases. Producer lineages can outcompete the sensitive lineage faster when cost of bacteriocin production is lower.

Figure 2

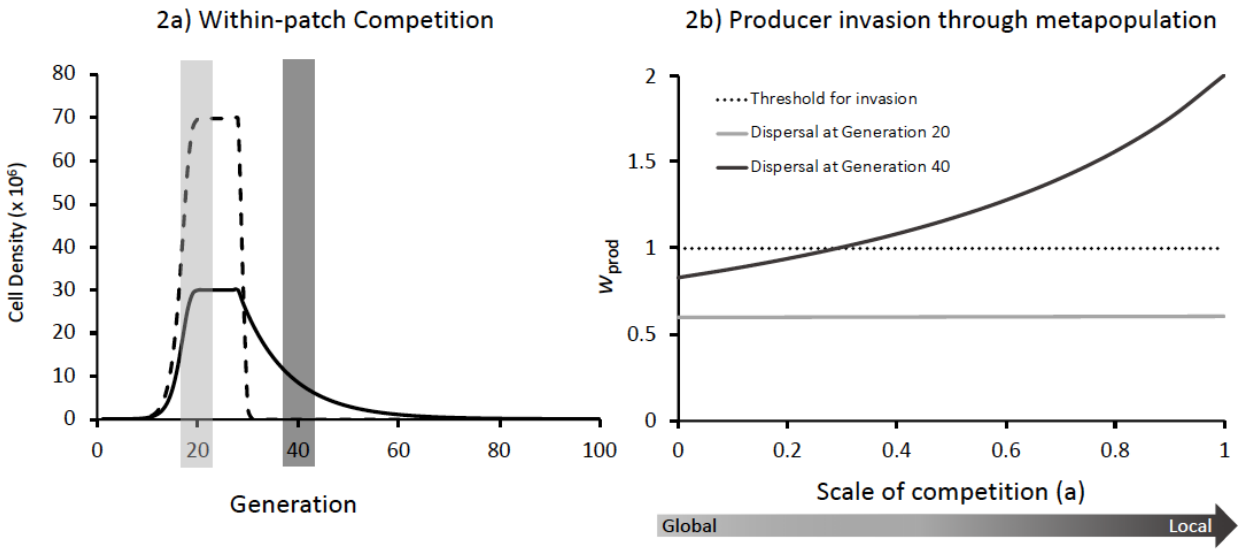

Figure 2: Model results showing within-patch competition (a) and invasion of producer through the metapopulation (b), when cost of bacteriocin production $x = 0.1$, and $S_{killed} = 10$. The black and grey bars in (a) represent dispersal times examined for invasion. The black and grey lines in (b) represent the relative fitness of the producer strain (w_{prod}) across varying levels of scale of competition, 'a', ranging from completely global (a = 0) to completely local (a =1). The dotted line in (b) represents the threshold w_{prod} value required for successful invasion which is 1. As seen in (b) the producer strain can invade the metapopulation if dispersal occurs after resources have been depleted (Dispersal at Generation 40) and there is some degree of local competition (a > 0.3).

Figure 3

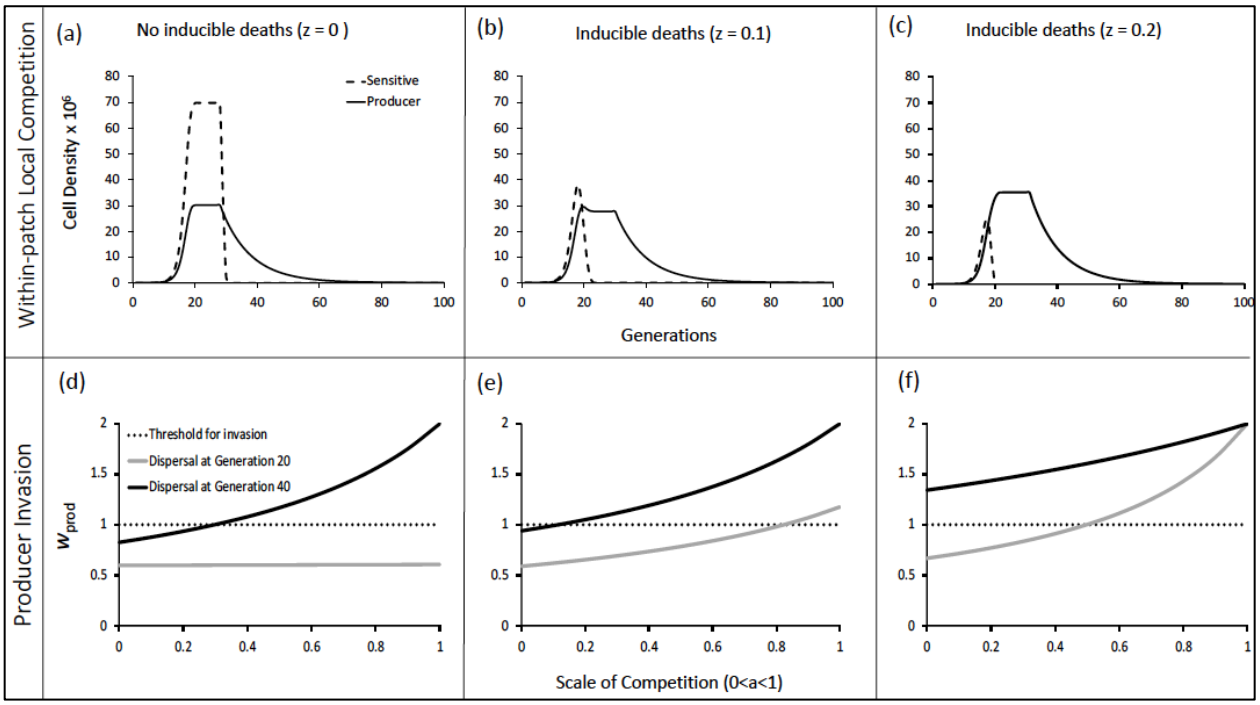

Figure 3: Model results examining within-patch competition (a-c) and invasion through the metapopulation (d-f) at varying levels of inducible deaths (z = 0, 0.1, and 0.2). Axes are the same as described in Figure 2. This model examines the consequences of phenotypically plastic bacteriocin release determined by the density and frequency of sensitive competitors in the focal patch. For all panels shown, x = 0.1, and S_{killed} = 10. Results suggest that incorporating plastic bacteriocin release can allow the producer strain to outcompete the sensitive strain faster (b, c) than they do when bacteriocin release only occurs upon natural deaths (a, z= 0) during within-patch competition. Inducible deaths promote the invasion of bacteriocin producers (e, f) relative to the lack of plasticity (d). At sufficiently high values of z, producers can invade even if dispersal occurs before resources are depleted (panel f, Dispersal at Generation 20).

Figure 4

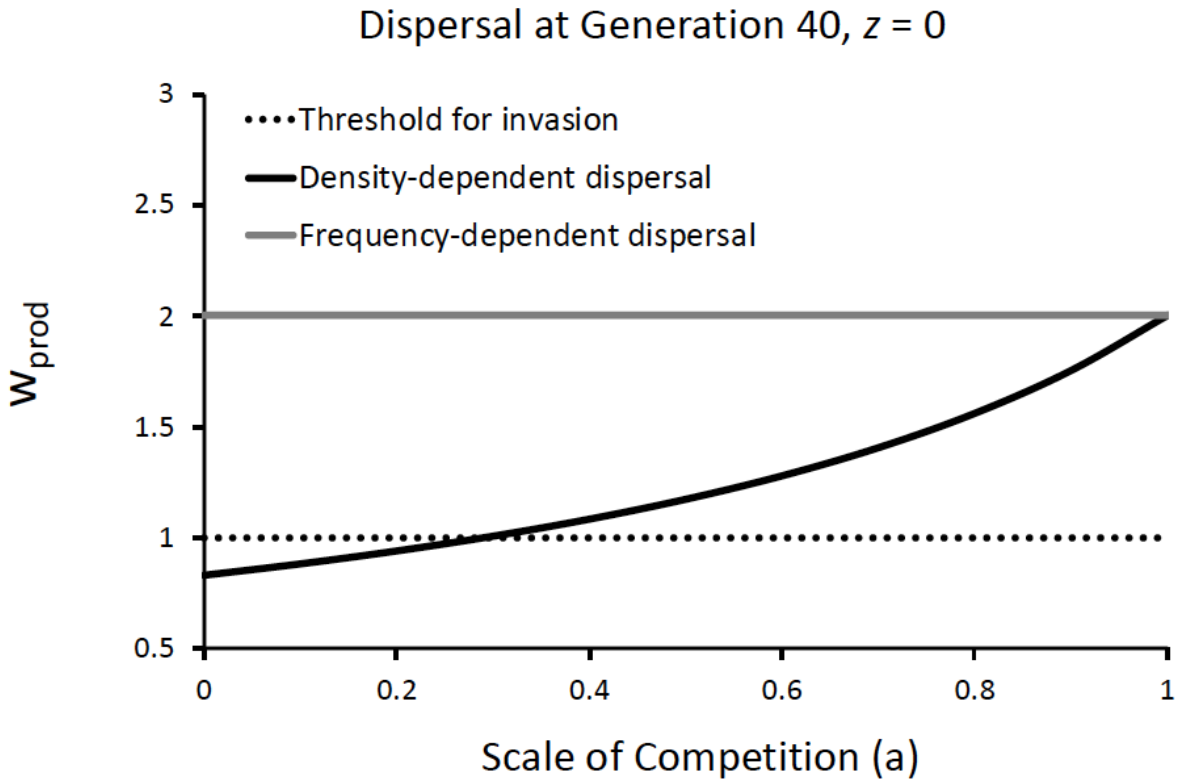

Figure 4: Model results showing how frequency-dependent dispersal and density-dependent dispersal may affect the invasion of producers through the metapopulation. If dispersal or transmission is limited by the number of vectors, as in the case of the entomopathogenic nematode *Xenorhabdus spp,* the invasion success of the producer depends on its relative frequency within the host instead of total density. As shown in the figure, under frequency-dependent selection, the consequences of scale of competition are lost. The figure shown here examines dispersal at Generation 40 only for z = 0.1 (see Figure 3e).

CHAPTER 3

Evolution of increased virulence is associated with decreased spite in the insect-pathogenic bacterium *Xenorhabdus nematophila*

A. Bhattacharya, V. C. Toro-Diaz, L. T. Morran and F. Bashey

Abstract

Disease virulence may be strongly influenced by social interactions among pathogens, both during the time course of an infection and evolutionarily. Here we examine how spiteful bacteriocin production in the insect-pathogenic bacterium *Xenorhabdus nematophila* is evolutionarily linked to its virulence. Theory predicts a negative correlation between virulence and spite due to their inverse correlations with pathogen growth. We examined bacteriocin production and growth across 14 experimentally evolved lineages that show faster host-killing relative to their ancestral population. Consistent with theory, these evolved, more virulent lineages showed reduced bacteriocin production and faster growth relative to the ancestor. Further, bacteriocin production was negatively correlated with growth across the examined lineages. These results strongly support an evolutionary trade-off between virulence and spiteful bacteriocin production and lend credence to the view that disease management can be improved by exploiting pathogen social interactions.

Introduction

The degree of damage caused by a pathogen to its host, i.e. the pathogen's virulence, is a complex trait determined by various factors. Key among these are the social interactions between the pathogens co-inhabiting a host (Frank, 1996; Leggett *et al.*, 2014). Pathogens exhibit myriad forms of social behaviors, such as cooperation and cheating (Foster, 2005; Köhler *et al.*, 2009). The direction of the effect of a given social behavior on virulence depends on how a behavior influences pathogen growth within the host (Gardner *et al.*, 2004; Buckling & Brockhurst, 2008). Faster growing pathogens are often assumed to be more virulent. Therefore, social traits that promote faster pathogen growth, such as the production of cooperative public goods may be positively correlated with disease virulence (Griffin *et al.*, 2004; Harrison *et al.*, 2006). On the other hand, social behaviors such as spite that can decrease the growth of pathogen populations are expected to be negatively correlated with virulence (Gardner *et al.*, 2004). Importantly, if these relationships have an underlying genetic basis, then the evolution of virulence may affect the evolution of social traits as well.

Almost all known lineages of bacteria exhibit the spiteful trait, bacteriocin production (Klaenhammer, 1988; Riley & Chavan, 2006). Bacteriocins are anticompetitor toxins that can kill closely related strains without killing clone-mates of the producer cells (Reeves, 1965). Bacteriocin production can reduce the growth of producing populations as bacteriocins are metabolically costly to produce and they are often released by cell lysis (Riley & Wertz, 2002; Riley & Chavan, 2006; Wloch-Salamon *et al.*, 2008). These negative effects on both the fitness of the producers and their sensitive competitors classifies bacteriocin production as a spiteful trait (West & Gardner, 2010). Theory predicts a negative correlation between bacteriocin

production and virulence as both the killing effect of bacteriocins and the growth costs of production can result in reduced pathogen densities, and thus, reduced virulence (Gardner *et al.*, 2004).

Empirical studies have shown that bacteriocin-mediated competitive interactions can reduce virulence during mixed infections. For instance, during co-infections with a sensitive competitor strain, the addition of bacteriocin-producing *Pseudomonas aeruginosa* lineages results in lower pathogen densities and thus, reduced virulence relative to bacteriocin non-producing mutants (Inglis *et al.*, 2009). Similarly, bacteriocin-meditated interactions among insect-pathogenic *Xenorhabdus spp.* within a host lead to reduced virulence (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Bashey *et al.*, 2012). However, evolutionary trade-offs between virulence and spite remain less well understood. Very few studies have examined the relationship between the evolution of spiteful traits and virulence evolution. However, Garbutt et al. (2011) found that evolution in mixed infections can result in reduced virulence and increased competitive antagonism relative to evolution in single infections in the insect-pathogen *Bacillus thuringiensis* (Garbutt *et al.*, 2011). Specifically, the increased antagonism occurred without a significant increase in absolute growth rates, suggesting that the observed antagonism was likely bacteriocin-mediated. Nonetheless, it is currently unclear if levels of bacteriocin production can be directly affected by selection on virulence.

Here, we examine the evolutionary interplay between virulence, spite and growth in the insect-pathogenic bacterium, *Xenorhabdus nematophila*. Using an experimental evolution approach, we ask whether the evolution of increased virulence is associated with changes in

spiteful bacteriocin production. We also examine the growth of the evolved and ancestral lineages to investigate the underlying correlation between growth and bacteriocin production.

Materials and Methods

Study System: *Xenorhabdus nematophila* is a species of insect-pathogenic bacteria that forms a mutualistic symbiosis with the nematode *Steinernema carpocapsae* (Martens *et al.*, 2003; Herbert & Goodrich-Blair, 2007; Stock & Blair, 2008). Free-living juvenile nematodes carry the bacteria in a specialized receptacle of the intestine, existing in a non-feeding state until they locate an insect host (Martens *et al.*, 2003). Within the insect, the nematode and bacteria separate, replicate, and quickly kill the insect host (Burman, 1982; Dunphy & Webster, 1988). Bacteriocin-mediated interactions between co-infecting *Xenorhabdus* strains are known to occur within the insect host (Morales-Soto & Forst, 2011).When host resources are depleted, the bacteria and nematodes re-associate to emerge and find a new insect-host (Martens *et al.*, 2003).

Bacterial Strains: Experimental evolution was conducted in a previous study (Morran *et al.*, 2016), where 16 independent lineages of *X. nematophila* derived from a single ancestral colony were passaged through *Galleria mellonella* caterpillar-hosts. In the next paragraph, we briefly describe the experimental evolution protocol used to generate these lineages. After 20 host passages, each bacterial lineage was injected into insect hosts and the mean time to host death was used as a measure of virulence (as shown in Figure 2a of (Morran *et al.*, 2016)). Pairwise comparisons between each evolved lineage and the ancestor revealed that 14 of the 16 lineages showed significantly faster host-killing than the ancestor (Figure 1a). These 14 evolved lineages were used in this study.

During every round of selection, each bacterial lineage was passaged by infecting 15 larval *G. melonella* caterpillars each with approximately 4000 CFUs. Depending on the treatment, the caterpillars were either infected with nematodes that carried the bacteria (M+), or directly injected with the appropriate bacterial dose by the experimenters (M-). Selection for faster host-killing (S+) was imposed by choosing only the first dead insect host for subsequent passaging. In contrast, in the 'S–' treatment, a dead host was chosen at random. Emerging juvenile nematodes were used for subsequent passaging in the 'M+' treatments. For 'M-' treatments, bacteria were extracted from the dead caterpillar hosts, serially diluted, and plated on selective NBTA agar plates with 50 μg/ml ampicillin (nutrient agar supplemented with 0.0025% (w⁄v) bromothymol blue and 0.004% (w⁄v) triphenyltetrazolium chloride, pH = 8). After 36 hours of growth, 20 colonies were picked and suspended in 1 ml phosphate buffered saline to repeat the infection process. A 20-colony slurry of the ancestral population was cryopreserved prior to experimental evolution, and similar 20-colony slurries of every experimental lineage were cryopreserved at the end of 20 host passages. Slurries were grown overnight in Luria-Bertani Broth (LB) at 28 ºC, and preserved as 20% glycerol solutions at -80 ºC. Additional details are available in the original paper (Morran *et al.*, 2016).

Bacteriocin extraction: Pure cultures of the ancestral and evolved lineages were grown in 5ml LB until OD_{600} = 0.5 at which point 0.5μg/ml Mitomycin C was added to chemically induce bacteriocins. After overnight incubation at 28º C, bacteriocin extracts were collected by centrifuging cultures at 1620 G for 5 minutes and filtering the supernatant through 0.45μm filters. This procedure allows the bacteriocin to pass through while eliminating any cells in the extract. Bacteriocin extracts were collected from one colony for every evolved lineage, and 4

colonies of the ancestral population. These extractions, as well as the growth measurements and bioassays described below, were conducted in 4 experimental blocks, such that 4 evolved colonies and 1 ancestral colony were processed simultaneously.

Growth measurements: The pure cultures used to extract bacteriocins were monitored for growth via periodic OD_{600} measurements on a spectrophotometer. OD_{600} values for all cultures after 4.5 hours of growth were used to compare growth rates. This time point was chosen as it was after all cultures reached exponential phase ($OD_{600} > 0.2$) and before Mitomycin C was added to the fastest growing culture.

Bacteriocin bioassay: The inhibitory activity of the bacteriocin extracts derived from the evolved and ancestral lineages were compared using a growth inhibition bioassay (Bhattacharya *et al.*, 2018). Briefly, a bacteriocin extract (or culture media in the 'no bacteriocin' controls) and a starting culture of sensitive cells (~10^6 CFU/ml) were mixed in 1:5 ratio by volume. Each mixture was pipetted into 4 replicate wells on a 100-well plate and incubated in a Bioscreen optical plate reader at 28 ºC with shaking at medium amplitude for 24 hours (GrowthCurves USA). Control wells with un-inoculated media were included to rule out contamination across the plate. OD_{600} measurements were taken every 30 minutes. These readings were used to calculate the lagtimes of the cultures growing in each well with the software GrowthRates 3.0 (Hall *et al.*, 2013).

The lagtime values of the cultures growing in the presence and absence of bacteriocin extracts were used to derive a metric of bacteriocin activity we call 'relative lag time'. The relative lag time is a ratio of the lagtime of a sensitive culture growing in the presence of

bacteriocin extracts and the lagtime of the respective 'no bacteriocin' control. Since the metric is a ratio, a value of 1 indicates no bacteriocin activity was detected. For every bacteriocin sample, the growth inhibition bioassay was conducted across two distinct strains, *Photorhabdus luminescens* TT01 and *X. bovienii* MC19, which are both sensitive to *X. nematophila* bacteriocin.

Statistical Analyses: All statistical analyses were performed using SAS 9.4. An analysis of variance was performed to conduct pairwise comparisons of 'mean time to host death' between the ancestor and the evolved lineages using Proc Mixed with 'Lineage' as a fixed effect. Bacteriocin activity (relative lag time) was compared using mixed-model analyses of variance with 'treatment' (Ancestral or Evolved), 'sensitive strain' (*P. luminescens* or *X. bovienii*), and their interaction as fixed effects; 'lineage' and 'experimental block' were included as random effects. Growth (OD_{600} at 4.5 hours) was analyzed similarly, with treatment and experimental block as factors. Pearson's correlation coefficient between bacteriocin production and growth was determined using Proc Corr. To evaluate the significance of this correlation, while accounting for the repeated bacteriocin measures (i.e. tested against two sensitive strains), Proc Mixed was used with 'relative lag time' as the dependent variable and 'OD' as a fixed effect; 'Experimental block' and 'Lineage' were included as random effects.

Results

The evolved lineages used here exhibited significantly faster host-killing, and thus, greater virulence relative to their ancestor (Figure 1a). Bacteriocin production in the evolved and ancestral lineages was tested against two sensitive strains using a growth inhibition bioassay. The evolved, more virulent lineages exhibited a significant decrease in 'relative lag time' ($F_{1, 15}$ =

15.30, $p < 0.001$) compared to the ancestral population (Figure 1b), indicating reduced bacteriocin production evolved over time. There was no significant effect of sensitive strain ($F_{1, 15} = 0.03$, $p = 0.864$) or interaction between treatment and sensitive strain ($F_{1, 15} = 0.04$, $p = 0.839$). Additionally, the evolved, more virulent lineages also show significantly higher OD_{600} values after 4.5 hours of growth ($F_{1, 13} = 4.73$, $p = 0.049$) relative to the ancestral strain (Figure 2a). Relative lag time and growth were negatively correlated across strains (Figure 2b), with greater growth coming at the expense of lower bacteriocin activity ($F_{1, 17} = 9.21$, $p = 0.008$). Thus, the evolution of increased growth rates and virulence was associated with the loss of bacteriocin production.

Discussion

Increasing evidence suggests that the virulence of pathogenic microbes may be affected by their social traits. Theory predicts a negative correlation between virulence and spite because virulence is often dependent on pathogen growth, and spite can result in lower growth of pathogenic populations (Gardner *et al.*, 2004). A premier example of spiteful behaviors in bacteria is the production of bacteriocins (Riley & Chavan, 2006). Here, we examined how the evolution of virulence affects growth and bacteriocin production using experimentally evolved lineages (Morran *et al.*, 2016) of the insect-pathogenic bacterium, *Xenorhabdus nematophila*. We found that the evolved, more virulent lineages (Figure 1a) exhibited reduced bacteriocin production and faster growth *in vitro* relative to their ancestor (Figure 1b, 2a). Further, bacteriocin production across all lineages showed a significant negative correlation with growth (Figure 2b).

Within-host competition among co-infecting pathogens can influence disease virulence in diverse ways. Increased competition for resources may select for faster pathogen growth and thus, increased virulence during mixed infections relative to single infections (de Roode *et al.*, 2005). But spiteful competitive interactions during mixed infections can reduce pathogen densities and select for reduced virulence (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Inglis *et al.*, 2009). Relatively little is known about how virulence and spiteful competition are evolutionary linked. Here we show that the evolution of increased virulence is associated with reduced bacteriocin production and faster growth in *X. nematophila*. It is important to note that during the course of experimental evolution these lineages did not encounter any bacteriocin-sensitive competitors. In the absence of competitors, selection for the maintenance of bacteriocin production should be weak, which can thereby facilitate the evolution of faster growth via reduced investment in bacteriocin production. Consistent with this idea, it has been shown that evolution during mixed-infections can select for increased antagonism and reduced virulence relative to single infections (Garbutt *et al.*, 2011). Taken together, these studies highlight the importance of competitive interactions in determining the evolutionary trajectory of pathogens (Bashey, 2015).

There has been considerable interest in exploring the relationship between virulence and social behaviors in pathogens for the possibility of exploiting it for therapeutic application (Foster, 2005; Brown *et al.*, 2009; Allen *et al.*, 2014). Cooperative bacterial traits such as public goods production and quorum sensing (Griffin *et al.*, 2004; Rumbaugh *et al.*, 2009) have received particular attention in this regard, as exploiting the ability of 'social cheats' to invade cooperative pathogen populations may reduce pathogen fitness and thus, virulence (Harrison *et*

al., 2006; Sandoz *et al.*, 2007). The tradeoffs between virulence and spite reported here, and as seen in reports of mixed infections (Massey *et al.*, 2004; Vigneux *et al.*, 2008; Garbutt *et al.*, 2011) suggest that maintaining selection for spite may help constrain the evolution of virulence. Akin to using 'social cheats' as therapeutic agents, perhaps the use of live competitors to maintain selection for bacteriocin production could help constrain pathogen growth and thus, reduce virulence. Genetically engineered probiotics that are being developed for therapeutic use (Sola-Oladokun *et al.*, 2017) may serve as potential candidates for such treatment strategies.

To conclude, we investigated how the evolution of increased virulence is associated with spiteful bacteriocin production and growth in insect pathogenic *X. nematophila*. Consistent with predictions, we found that the evolution of increased virulence is associated with reduced bacteriocin production and faster growth *in vitro*. Such trade-offs between virulence and spite may offer novel opportunities to exploit social traits in pathogenic populations for disease management. These results provide some of the earliest direct insights into how the evolution of virulence and bacteriocin production are correlated and demonstrate how the social lives of pathogenic bacteria may be intricately linked with disease.

Acknowledgements: We thank McKenna Penley for logistical support and gratefully acknowledge CISAB at Indiana University for funding to AB and VCTD. We thank C. M. Lively for supporting the experimental evolution study from which the experimental lines were obtained.

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Figure 1

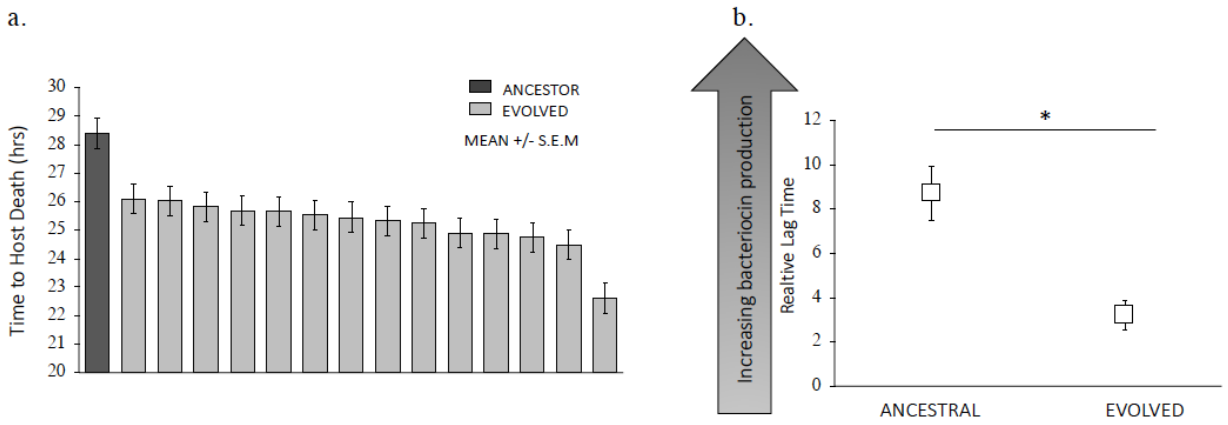

Figure 1: Increased virulence (a) of the14 evolved lineages (grey bars) relative to the ancestor (black bar) is shown as a lower mean (+/- s. e.) time to host death. Reduced bacteriocin production (b) is shown as a lower mean (+/- s. e.) relative lag time, indicating that the bacteriocin extracted from the evolved lineages was less inhibitory than the bacteriocin extracted from the ancestor.

Figure 2

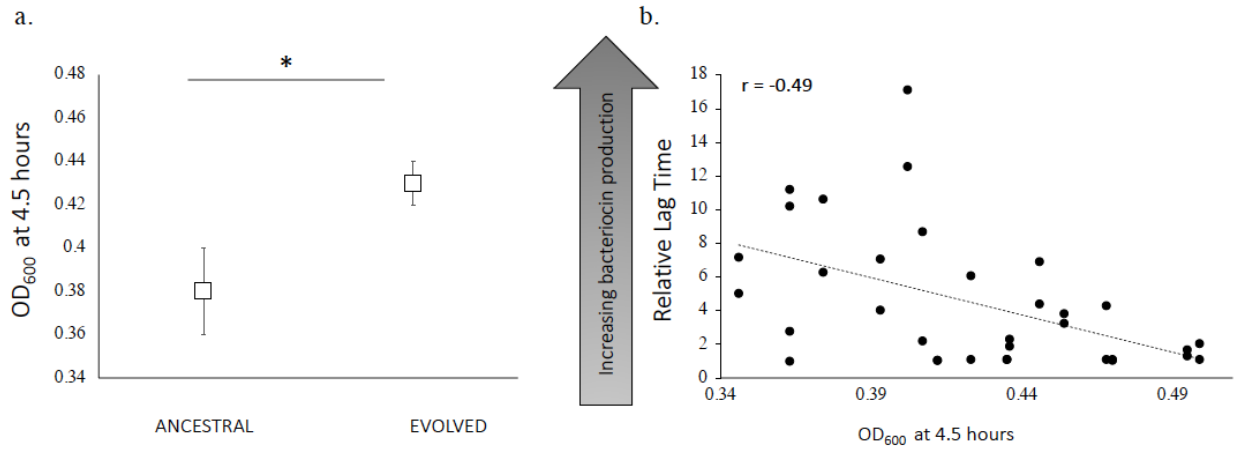

Figure 2: Evolved, more virulent lineages grow faster than the ancestor (a) and show a significant negative correlation with bacteriocin production (b). Growth is shown as mean (+/- s. e.) OD_{600} after 4.5 hours culturing *in vitro*. Bacteriocin production is shown as the relative lag time value exhibited by all evolved and ancestral lineages against both sensitive strains. Pearson's correlation coefficient, r = -0.49.

CHAPTER 4

Suppression of bacteriocin resistance using live, heterospecific competitors

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Abstract

Rapidly spreading antibiotic resistance has led to the need for novel alternatives and sustainable strategies for antimicrobial use. Bacteriocins are a class of proteinaceous anticompetitor toxins under consideration as novel therapeutic agents. However, bacteriocins, like other antimicrobial agents, are susceptible to resistance evolution, and will require the development of sustainable strategies to prevent or decelerate the evolution of resistance. Here we conduct proof-of-concept experiments to test whether introducing a live, heterospecific competitor along with a bacteriocin dose can effectively suppress the emergence of bacteriocin resistance *in vitro*. Previous work with conventional chemotherapeutic agents suggests that competition between conspecific sensitive and resistant pathogenic cells can effectively suppress the emergence of resistance in pathogenic populations. However, the threshold of sensitive cells required for such competitive suppression of resistance may often be too high to maintain host health. Therefore, here we aim to ask whether the principle of competitive suppression can be effective if a heterospecific competitor is used. Our results show that a live competitor introduced in conjunction with low bacteriocin dose can effectively control resistance and suppress sensitive cells. Further, this efficacy can be matched by using a bacteriocin-producing competitor without any additional

bacteriocin. These results provide strong proof-of-concept for the effectiveness of competitive suppression using live, heterospecific competitors. Currently in-use probiotic strains or commensals may provide promising candidates for the therapeutic use of bacteriocin-mediated competitive suppression.

Introduction

Rapidly spreading antibiotic resistance has created an urgent need to find novel antimicrobials (Projan & Shlaes, 2004; Allen *et al.*, 2014) and usage strategies that can constrain the emergence of resistance (Olofsson & Cars, 2007; Read *et al.*, 2011). One potential source of alternative antimicrobials is bacteriocins, which are proteinaceous toxins produced by bacteria, noted for their ability to kill closely related strains (Cotter *et al.*, 2013). Bacteriocins are ubiquitously produced by almost all known lineages of bacteria, (Klaenhammer, 1988; Riley & Chavan, 2006) increasing their appeal as potential alternatives to replace antibiotics. However, resistance against bacteriocins has been frequently reported in natural as well as clinical isolates (Gordon *et al.*, 1998; Hawlena *et al.*, 2010b; Koch *et al.*, 2014), and relative to conventional antibiotics, little is known about the evolution of resistance against bacteriocins (Inglis *et al.*, 2016). Here we examine the evolution of resistance against bacteriocins in the insect-pathogenic bacteria *Xenorhabdus bovienii* and conduct *in-vitro* experiments to investigate a novel strategy for preventing the emergence of resistance.

The traditional approach of aggressive chemotherapy used with conventional antimicrobials employs high doses with the goal of eliminating all pathogen cells including partially resistant mutants (Ehlrich, 1913). However, by eliminating all sensitive cells, this

approach imposes strong selection favoring resistant mutants that may be present at the onset of infection or be acquired over the course of infection (Read *et al.*, 2011; Day & Read, 2016). Recent studies have proposed a 'moderate chemotherapy' or 'containment' approach that is more effective at suppressing resistant mutants from spreading. Moderate chemotherapy employs lower antimicrobial doses than aggressive approaches and does not eliminate all sensitive pathogenic cells. Competition between the untreated sensitive cells and resistant mutants results in competitive suppression of resistance and thereby prevents the spread of resistant mutants (de Roode *et al.*, 2004; Kouyos *et al.*, 2014; Hansen *et al.*, 2017; Wale *et al.*, 2017). However, the success of this approach depends critically on maintaining a threshold density of sensitive cells for competitive suppression of resistance to occur. If this threshold is too high for a host to tolerate without succumbing to the infection, moderate chemotherapy will be ineffective. Under such circumstances, aggressive approaches are recommended (Hansen *et al.*, 2017), which can be problematic as they impose strong selection for resistance. Thus, for sustainable use of antimicrobials, it is necessary to find strategies that can simultaneously achieve two major goals: (a) effective suppression of pathogen cell densities, and (b) prevention of resistance evolution.

Over the course of debates on the relative efficacy of aggressive versus moderate treatment strategies, competition between drug-sensitive and drug-resistant pathogenic cells has repeatedly surfaced as a key determinant of the emergence and spread of resistance (de Roode *et al.*, 2004; Huijben *et al.*, 2010; Colijn & Cohen, 2015; Day *et al.*, 2015). Although models specifically emphasize competition between conspecific drug-sensitive and drug-resistant cells, the conceptual framework that underpins the role of competition in constraining resistance spread should be applicable to any ecological competitor. In other words, any strong competitor,

whether it is a conspecific sensitive strain or heterospecific neighbor, may impose similar constraints on the spread of resistant mutants of the target strain if the competitor itself is unaffected by the antimicrobial used.

Here, we examine whether the conceptual basis of competitive suppression of resistance can be extended to include heterospecific competitors. We examine the evolution of resistance against bacteriocins and conduct *in-vitro*, proof-of-concept experiments to determine whether using a heterospecific competitor strain in conjunction with bacteriocin dose, can constrain the spread of resistant mutants in the population while also driving down densities of bacteriocin-susceptible pathogenic cells. We expect high bacteriocin doses to impose selection favoring resistant mutants. In contrast, we predict that incorporating a heterospecific competitor with the high bacteriocin dose will reduce the emergence of resistance as the additional competitive challenge will impede the spread of resistant mutants. We further predict that low bacteriocin doses will result in lower selection for resistance than high doses; however, low doses may not effectively suppress total cell densities. We predict that using a competitor with low bacteriocin doses will not only slow the evolution of resistance but will also suppress untreated sensitive cells resulting in a better overall outcome relative to using the low dose without a competitor. Additionally, we predict that using only a competitor that itself produces a low dose of the bacteriocin should be effective at reducing total and resistant cell densities of the focal, target strain. Finally, these predictions rely on the assumption that resistance imposes a cost that makes resistant mutants inferior competitors to their sensitive counterparts. Thus, to determine whether resistance is indeed costly in our experiments, we also compare the growth of resistant mutants derived from bacteriocin-exposed populations with the growth of sensitive cells.

Materials and Methods

Experimental Design: To examine the effect of dose on the growth of sensitive cells and evolution of bacteriocin resistance, cultures of a bacteriocin-sensitive strain, *Xenorhabdus bovienii,* were exposed to two different doses of bacteriocin (high and low) and a 'no bacteriocin' control. The effect of competitive suppression was examined by either introducing a live competitor strain in conjunction with the bacteriocin dose ('high dose + competition' and 'low dose + competition') or exposing cells to bacteriocin alone. Finally, the effect of a bacteriocin-producing competitor alone was examined by introducing a competitor that produces a low dose of the bacteriocin to the cultures of the sensitive strain (bacteriocin-producing competitor). In all treatments, we determined both total and resistant cell densities of the focal, target strain *Xenorhabdus bovienii*.

Bacterial Strains: A natural isolate of the bacteriocin-sensitive strain *Xenorhabdus bovienii*, Bov59 (isolated as described in Hawlena *et al.*, 2010b), was used as the focal, target strain. Both high and low doses of bacteriocin were derived from a natural isolate of *X. koppenhoefferi*, Kop46 (isolated as described in (Hawlena *et al.*, 2010a)), which is also a sympatric competitor of the bacteriocin-sensitive strain (Hawlena *et al.*, 2012). The competitor used in the 'high dose + competitor' and 'low dose + competitor' treatment was a mutant *X. koppenhoefferi* strain (Kop46 mut) that does not release functional bacteriocin (construction as described in Morales-Soto & Forst, 2011). For the 'bacteriocin-producing competitor' treatment, the wild type Kop46 strain was used. These strains of *X. koppenhoeferi* and *X. bovienii* are morphologically distinct whereby Kop46 colonies appear maroon and Bov59 colonies appear blue on NBTA plates (nutrient agar supplemented with 0.0025% (w/v) bromothymol blue (Sigma Aldrich) and

0.004% (w/ v), 5 triphenyltetrazolium chloride (Sigma Aldrich), pH = 8). In addition, Bov59 has a higher natural resistance to ampicillin than Kop46, thereby enabling further distinction on NBTA plates with 75μg/ml ampicillin. All cultures used in the experiment were derived from freezer stocks maintained at -80ºC and streaked onto NBTA plates prior to each replicate.

Bacteriocins: Bacteria in the genus *Xenorhabdus* produce a phage tail-like bacteriocin, xenorhabdicin (Boemare *et al.*, 1992; Thaler *et al.*, 1995). Like most bacteriocins, xenorhabdicin has a narrow killing range, affecting other *Xenorhabdus* strains and strains in the closely related genera *Photorhabdus* and *Proteus*. Xenorhabdicin is similar in structure to R-type pyocins, where killing requires attachment of tail fibers to the target cell and results from a puncture and subsequent depolarization of the cell membrane (Williams *et al.*, 2008). Xenorhabdicin is encoded by a remnant P2 phage cluster, approximately 30 kb in length (Morales-Soto & Forst, 2011; Morales-Soto *et al.*, 2012; Ciezki *et al.*, 2017). Growth inhibition of the target strain Bov59 by the xenorhabdicin produced by Kop46 was determined by insertional inactivation of the sheath gene and subsequent lack of inhibitory phenotype observed in the mutant phenotype (Bashey, Forst, and Palmer, unpublished data).

Bacteriocin Doses: Both high and low doses of the bacteriocins used were procured from wild type Kop46 cultures. High dose bacteriocin extracts were collected from Kop46 cultures that were chemically induced with Mitomycin C (Sigma Aldrich). Briefly, pure cultures of Kop46 cultures in exponential phase were incubated with 0.5μg/ml Mitomycin C. After overnight incubation at 28º C, bacteriocin extracts were collected by centrifuging cultures at 1620 G for 5 minutes and filtering the supernatant through 0.45μm filters (Acrodisc). This procedure allows

the bacteriocin to pass through while eliminating any cells in the extract. Previous work with this isolate has shown that PEG precipitations of filtered supernatants (to further isolate the phage tail-like bacteriocins) show identical patterns of inhibitory activity as the un-precipitated doses used here, when tested against over 10 different target genotypes. Further, no active phage has been found in the strains used in this study (Hawlena *et al.*, 2010a).

The low dose was obtained from stationary phase cultures of Kop46 without chemical induction. This choice of the low dose of bacteriocin was deliberate to ensure that the amount of bacteriocin produced by the competitor in the 'bacteriocin-producing competitor only' treatment matched the low dose used in the 'low dose + competition' treatment. This is further confirmed by earlier work showing that the concentration of bacteriocin produced by Kop46 is not affected by co-culturing with Bov59 (Bhattacharya *et al.*, 2018). Finally, the inhibitory profile of Kop46 bacteriocin with and without chemical induction was identical in 9 tested strains, indicating that the same antimicrobial is released at both doses used in the experiment. All bacteriocin extracts were stored at 4º C until used.

Bioassay to quantify high and low bacteriocin doses: A growth inhibition bioassay (previously described in Bhattacharya *et al.*, 2018) was used to quantify and compare the high and low doses of bacteriocin used in the experiments. This bioassay compares the inhibitory effect of different bacteriocin doses by comparing the duration of lag imposed on the growth of a sensitive strain by fixed amounts of the bacteriocins. To do this, we mixed a bacteriocin dose (or culture media in the 'no bacteriocin' control) and a starting culture of sensitive cells in 1:5 ratio by volume. The growth of the sensitive strain in the presence and absence of bacteriocin doses

was measured on a Bioscreen optical plate reader (GrowthCurves USA). The doses examined in this experiment included the chemically induced 'high dose', three 10-fold serial dilutions of the high dose, and the 'low dose' derived from chemically uninduced Kop46 cultures. Four replicate wells of each bacteriocin dose were examined in a 100-well plate reader (Honeycomb plate, Growth Curves USA). Each well contained 200µl of the respective bacteriocin + culture mixture at an initial density of 10^6 CFU/ml. Control wells with un-inoculated media were included to rule out contamination. The plates were incubated in the optical plate reader at 28° C with continuous shaking at medium amplitude, and OD_{600} was recorded every 30 min for 24 hours.

The OD_{600} data was used to generate growth curves, and the lag time of the cultures growing in each well was calculated using the software GrowthRates 3.0 (Hall *et al.*, 2013). Lag time represents the time taken by a starting culture to reach exponential growth phase. The inhibitory activity of bacteriocins results in increased lag times of cultures that are exposed to bacteriocins relative to negative control cultures that are not exposed to bacteriocins (Figure 1). This increase in lag time provides a metric of bacteriocin inhibitory activity and thus dose. A growth inhibition bioassay revealed that the lag times of Bov59 cultures exposed to the 'high dose' bacteriocin were significantly longer than the lag times of cultures exposed to 'low dose' bacteriocin ($F_{1,18} = 62.41$, $p < 0.0001$, Figure 1).The 'low dose' bacteriocin imposed significantly greater growth inhibition than the 'no bacteriocin' control ($F_{1,18} = 130.64$, $p < 0.0001$), 1:1000 dilution ($F_{1,18} = 132.25$, $p < 0.0001$), 1:100 dilution ($F_{1,18} = 94.09$, $p < 0.0001$) and significantly less inhibition than the 1:10 dilution ($F_{1,18} = 27.45$, $p < 0.0001$) bacteriocins (Figure 1).

Experimental protocol to determine the effects of dose and competition: Starting cultures (~10^6 CFU/ml) of bacteriocin-sensitive Bov59 were established by inoculating 5ml LB media (Difco) with 100µl of an overnight culture in 20ml culture tubes. To examine the effect of dose on the evolution of bacteriocin resistance, cultures were either exposed to 1ml of bacteriocin dose (high or low), or 1ml additional LB ('no bacteriocin' control). The effect of competitive suppression was examined by either introducing a live competitor strain (Kop46 mut) in conjunction with the bacteriocin dose or exposing cells to bacteriocin alone. The mutant Kop46 strain was co-inoculated in the competition treatment tubes (50µl of an overnight culture), in addition to the respective bacteriocin dose. Finally, the effect of a bacteriocin-producing competitor alone was examined by directly co-inoculating 50ul of overnight cultures of a bacteriocin-producing competitor (Kop46) without any additional bacteriocin dosage. All cultures were incubated for 24 hours at 28º C with shaking at 120 rpm (New Brunswick C2 platform shaker, Easton NJ). Nine independent experimental replicates of all the treatments were performed.

After 24 hours, serial dilutions were plated to estimate total and resistant cell densities of the focal, target strain Bov59, as well as, densities of the heterospecific competitors. Total density of the Bov59 cells was estimated by plating 100ul of the culture dilutions on NBTA agar plates with 75µg/ml ampicillin (NBTA+Amp75). Both bacteriocin-sensitive and bacteriocin-resistant Bov59 cells can grow on these plates but the heterospecific competitor used in the experiment does not grow on these plates. Moreover, the Kop46/Kop46 mutant colonies are morphologically distinct from Bov59 allowing the colony counts of each species to be further confirmed.

To determine resistance, 100ul aliquots of the dilutions were mixed with equal volumes of chemically induced bacteriocin before plating (as in Bashey, Young, Hawlena, & Lively, 2012) allowing only resistant colonies to grow. This method of measuring resistance evolution was independently validated as is described in following section. Colony counts for total Bov59 and bacteriocin-resistant Bov59 determined from these plates were used to calculate the proportion of resistant mutants in each treatment. Additionally, one dilution was plated on NBTA agar plates without ampicillin which is a non-selective media plate and thus confirmed the absence of contamination in cultures. In all cases where no colonies were detected on a plate, the detection limit of cell density for that plate was used instead of zero, to be as conservative with estimates as possible.

Growth inhibition bioassay to validate resistance evolution: To validate the method used for detecting resistance evolution, 10 colonies from one replicate of the 'high dose' treatment were chosen at random. These 10 test colonies were derived from a NBTA+Amp75 plate used to calculate total Bov59 density. The colonies were picked, inoculated in 5ml LB media overnight and then used to conduct a growth-inhibition bioassay as described above in the Bioassay section. All 10 test colonies, and a bacteriocin-sensitive, wild type Bov59 culture were grown in the presence and absence of chemically induced bacteriocin. Starting cultures were mixed with bacteriocin or additional growth media in a 5:1 ratio, and growth of the cultures was measured in an optical plate reader. OD_{600} readings were taken every 30 minutes for 24 hours. The resulting growth curves were examined to look for bacteriocin-mediated inhibition. Typically, sensitive cultures show strong bacteriocin-mediated inhibition of growth depicted by increased lag times in the presence of bacteriocin relative to the absence of bacteriocin (as shown in Figure 1).

Resistance was determined as the lack of strong inhibition in the presence of bacteriocin. Among the examined colonies, 9 out of 10 showed resistance. The resistant colonies showed a significantly shorter lag time when exposed to bacteriocin (mean +/- S.E.M = 42.62 +/- 7.9 minutes) relative to the sensitive cultures (mean +/- S.E.M = 1263.71 +/- 46.68 minutes, t= 25.79, df = 1, p = 0.02) The proportion of resistant colonies (9 out of 10, i.e. 90%) was consistent with the proportion calculated for that replicate (89.13%) by comparing Bov59 CFUs from plating with and without bacteriocin, thereby validating the experimental methods used to measure resistance evolution.

Cost of resistance: To determine whether the evolution of resistance against bacteriocins is associated with growth costs, growth parameters while growing in the absence of bacteriocin were compared for sensitive and resistant colonies isolated from two independent experiments. First, we examined the 10 test colonies derived from the current experiment described in the previous paragraph. Growth parameters in the absence of bacteriocin for the 9 resistant colonies were compared to growth parameters of the sensitive colony and a wild type Bov59 colony. OD_{600} values for four replicate wells for each colony were measured by the Bioscreen optical plate reader and were used to estimate colony average growth rate and maximum OD values by the software GrowthRates 3.0 (Hall *et al.*, 2013). These colony averages were used to compare growth between resistant and sensitive colonies.

Resistant colonies were also isolated from an independent experiment. In this second experiment, initial cultures of wild-type Bov59 were grown in the presence (Exposed) and absence (Negative) of a high dose of bacteriocin in a 100-well microtiter plate (Honeycomb

plate, Growth Curves USA). Four independent replicates from each condition were pooled and serial dilutions were plated on NBTA agar plates. Five separate colonies from each treatment (Exposed and Negative) were preserved by growing overnight in LB and making freezer stocks in glycerol, which were stored at -80ºC. The freezer stocks were used to conduct growth inhibition bioassay as described above. All five 'Exposed' stocks showed resistance to bacteriocins, while all five 'Negative' treatment stocks showed bacteriocin sensitivity. Growth parameters of these colonies in the absence of bacteriocin were determined as described in the previous paragraph.

Statistical analysis: The effect of different bacteriocin doses on the lagtimes of a sensitive Bov59 cultures were compared by performing a one-way analysis of variance using 'bacteriocin dose' as a fixed effect in Proc Mixed. To compare the effect of bacteriocin dose and the presence of a competitor on $\log_{10}$-transformed cell densities and proportion resistance values, mixed model analyses of variance with 'bacteriocin dose' and 'competition' as fixed effects and 'experimental block' as random effect were used in Proc Mixed. Bacteriocin-induced delay in lagtime, growth rates, and maximum OD values of sensitive and resistance cultures were compared using Proc Ttest with the Satterthwaite approximation to account for unequal variance and sample sizes. To visualize growth of resistant and sensitive colonies, a LOESS regression on the colony average OD was used to estimate the mean OD per time for each type of colony grown. All analyses were performed in SAS 9.4.

Results

Exposure to high bacteriocin dose reduces total Bov59 densities but imposes strong selection for resistance: Exposure to a high dose of bacteriocin resulted in significantly lower total Bov59 cell densities (Figure 2A) than the 'no bacteriocin' ($F_{1,16} = 87.23$, $p < 0.0001$) and 'low-dose bacteriocin' treatments ($F_{1,16} = 83.53$, $p < 0.0001$). Specifically, the high dose treatment had over an order of magnitude reduction in total Bov59 densities (high dose CFU/ml = 6.6×10^6 vs $7.8 \times 10^7$and 7.1×10^7 CFU/ml in the 'no bacteriocin', 'low dose' treatments, respectively). While this shows the effectiveness of the high dose in reducing total cell densities, exposure to the high dose of bacteriocin also imposed strong selection for bacteriocin resistance (Figure 2B). The resistant Bov59 density was 35- to 62-fold higher in the 'high dose' treatment (5.1×10^6 CFU/ml) relative to the 'no bacteriocin' (mean CFU/ml = 2.1×10^5, $F_{1,16} = 46.51$, $p = 0.0001$) and 'low dose' (mean CFU/ml = 5.1×10^5, $F_{1,16} = 15.52$, $p = 0.0012$) treatments. Although the 'low dose' treatment had twice as many resistant cells as the 'no bacteriocin' treatment, this difference was not statistically significantly ($F_{1,16} = 1.08$, $p = 0.31$). Total Bov59 densities measured in the low bacteriocin treatment were also not significantly different from the no bacteriocin treatment ($F_{1,16} = 0.05$, $p = 0.82$). It was confirmed, however, that the low bacteriocin treatment showed inhibitory activity against the target strain, using a growth inhibition assay (Figure 1).

Incorporating a live, heterospecific competitor with bacteriocin dose reduces total and resistant cell densities: To examine the effectiveness of competitive suppression, a live competitor strain was introduced simultaneously with the bacteriocin doses. The addition of a

competitor significantly reduced the total ($F_{1,24} = 45.2$, $p < 0.0001$) and resistant Bov59 densities ($F_{1,24} = 254.7$, $p < 0.0001$) relative to the use of bacteriocins only at both doses (Figure 3). Further, adding the Kop46 mutant competitor reduced the percentage of resistant cells in the high-dose treatment from 83.6 % to 0.3 % ($F_{1,24} = 128.14$; $p < 0.0001$), demonstrating that competition prevented resistant mutants from spreading in the population. For the low dose treatment percent resistance was 0.9% without the competitor. No resistant colony was detected in any of the nine replicates of the 'low dose + competitor' treatment, at a detection limit of 100 CFU/ml; although, total Bov59 density was on the order of $\sim 10^5$ CFU/ml for this treatment. Overall, the 'low dose + competitor' treatment yielded the lowest total and resistant cell densities in the target strain (Figure 3).

Despite the overall reduction in total cell densities upon the addition of a live, heterospecific competitor, the 'high dose + competitor' treatment maintained a significantly higher density of Bov59 cells (mean CFU/ml = 1.8×10^6) relative to the 'low dose + competitor' treatment (mean CFU/ml = 6.5×10^3, $F_{1,24} = 01.04$; $p < 0.0042$). Such high total densities of Bov59 in the 'high dose + competition' treatment may reflect the escape of resistant mutants, despite a strong reduction in percent resistance upon the addition of the Kop46 mutant competitor. The density of resistant cells in the 'high dose + competitor' treatment (mean CFU/ml = 1999.11) was significantly higher than the resistant cell density in the 'low dose + competitor' treatment (mean CFU/ml = 99 , $F_{1,24} = 15.92$; $p = 0.0005$). For replicates where no resistant colonies were found, the resistant cell density calculations used the detection limit instead of zero to be conservative. While no resistant colonies were detected in the 'low dose +

competition' treatment resulting in mean CFU/ml = 99 which was the detection limit, resistance was detected in one replicate of the 'high dose + competition' treatment at a density of 10000 CFU/ml suggesting that the likelihood of resistance escape was higher in the latter treatment. The mean density of the Kop46 mutant competitor itself was 0.9 x 10^6 CFU/ml in the 'high dose + competitor' treatment and 1.2 x 10^8 CFU/ml in the 'low dose + competitor' treatment.

Competition with bacteriocin-producing competitor alone effectively suppresses total and resistant Bov59 densities: We examined whether using a bacteriocin-producing strain, which releases bacteriocin matching the concentration of the low-dose treatment, could be just as effective as the 'low dose + competitor' treatment. Total and resistant cell densities of the target strain in this treatment (Figure 3) show that this approach is as successful as the low dose with competition treatment in suppressing target cells ($F_{1,8} = 2.57$, $p = 0.15$) and constraining resistance ($F_{1,8} = 1.0$, $p = 0.35$). No resistant colonies were detected in 8 out of the 9 replicates examined at a detection limit of 100 c.f.u/ml. The remaining replicate showed 0.5% resistance. The mean density of the Kop46 competitor itself was 1.2 x 10^8 CFU/ml.

Resistant mutants pay a growth cost relative to sensitive cells: To determine whether resistant mutants pay a cost of resistance, we compared the growth in the absence of bacteriocin of resistant and sensitive colonies derived from two independent experiments. Nine resistant colonies from one "high dose" replicate of the main experiment were compared to the growth of the single sensitive culture from that replicate and a wild type Bov59 culture. Cultures derived from resistant colonies showed significantly reduced growth (Figure 4A, specifically they have

lower growth rates ($t = 3.87$, $df = 9$, $p = 0.003$) and reach lower maximum OD values ($t = 5.29$, $df = 9$, $p = 0.0004$). Growth of 5 bacteriocin-resistant and 5 bacteriocin-sensitive cultures derived from a separate experiment also show distinct patterns in the absence of bacteriocin (Figure 4B). Again, consistent with a cost of resistance, bacteriocin-resistant lineages show significantly reduced growth rates ($t = 3.05$, $df = 4$, $p = 0.037$) and a statistical trend towards lower maximum OD values ($t = 2.48$, $df = 4$, $p = 0.06$) relative to the bacteriocin-sensitive lineages.

Discussion

Conventional chemotherapeutic methods involving the use of high antibiotic doses may be effective initially, but as they impose strong selection favoring resistant mutants they ultimately result in treatment failure. Using lower doses can enable competitive suppression of resistance, when the host can tolerate susceptible pathogen loads that are sufficient to outcompete resistant mutants. However, using lower doses may not be feasible when the host cannot tolerate sufficient susceptible pathogenic cells to compete with resistant mutants. Here, we conduct *in vitro* experiments to test whether the principle of competitive suppression can be employed by using a heterospecific competitor in conjunction with antimicrobial dose to suppress resistance evolution, and overall pathogen load. We test this concept using bacteriocins and natural isolates of bacteriocin-producing *Xenorhabdus* populations. Our results suggest that incorporating a live competitor strain in conjunction with bacteriocin can significantly reduce the density of resistant cells. We find that the combination of low dose bacteriocin and a heterospecific competitor is most effective at suppressing overall cell densities and the evolution of resistance against bacteriocins (Figure 3). We demonstrate that the same efficacy may be achieved by using a bacteriocin-producing competitor strain which releases a low dose of the bacteriocin and

simultaneously competes with the susceptible population. Finally, we find that the evolution of resistance against bacteriocins *in vitro* is associated with significant growth costs detectable in the growth rates as well as yield in cultures of resistant lineages relative to sensitive lineages (Figure 4). These results provide strong proof-of-concept for the effectiveness of employing competitive suppression to control the spread of bacteriocin resistance while also lowering susceptible cell densities.

This study examines the evolution of resistance against bacteriocins, which are anticompetitor toxins ubiquitously produced by bacteria. Bacteriocins have been proposed as alternatives to replace the fast-depleting pool of antibiotics. In addition to being noted for their efficacy against pathogenic strains both *in vitro* (Noll *et al.*, 2011; Sandiford & Upton, 2012) and *in vivo* (Goldstein *et al.*, 1998; Kruszewska *et al.*, 2004; De Kwaadsteniet *et al.*, 2009), bacteriocins demonstrate low cytotoxicity to host cells (Maher & McClean, 2006; Jasniewski *et al.*, 2009), contributing to their suitability as therapeutics (reviewed in Cotter et al., 2013; Riley et al., 2012). Bacteriocins typically have narrow killing spectra; although, some broad-spectrum bacteriocins are known (McAuliffe *et al.*, 1998; Riley & Chavan, 2006; Lim *et al.*, 2016). In the search for commercially viable therapeutics, broad killing spectra may seem appealing due to the wide range of pathogens one agent can treat. However, the potential risk of disrupting commensal microbial community within the host, and the increased potential for the evolution of resistance against the agent, may be important reasons to make narrow spectrum bacteriocins the favorable choice for sustainable therapeutic application (Rea *et al.*, 2011). Further, the narrow-killing range of bacteriocins may make them more amenable to the reduction of resistance evolution via the competitive suppression mechanism explored in our study as a resistant

competitor strain is required. Although bacteriocins have been less well-exploited as therapeutics, bacteriocin-production is known to play a key role in the efficacy of probiotic supplements. Many probiotic bacteria confer health benefits via bacteriocin-mediated competition resulting in their establishment in the microbiome (as reviewed by (Gillor *et al.*, 2009; Dobson *et al.*, 2012). Additionally, some studies have found strong evidence to suggest that bacteriocin-producing probiotic strains can effectively inhibit pathogenic strains including enterohaemorrhagic *Escherichia coli* and *Listeria monocytogenes* (Su *et al.*, 2007b; a), further highlighting the potential for using bacteriocins as alternative therapeutics to replace antibiotics.

However, this potential may be squandered if bacteriocins are used in a conventional chemotherapeutic approach. Our results demonstrate that exposure to high doses of bacteriocin can lead to rapid evolution of resistance (Figure 2). In contrast, using a heterospecific competitor in conjunction with a high bacteriocin dose can suppress the spread of resistant mutants (Figure 3). The percent of resistant cells in the target population when a heterospecific competitor is added drops to 0.3% as compared to 87.6% in the presence of high dose without a heterospecific competitor. This striking reduction in the proportion of resistant cells when a competitor is present suggests that the heterospecific competitor was disproportionately constraining the spread of resistant mutants relative to sensitive cells. This observation is consistent with the hypothesis that resistant mutants pay costs of resistance that make them inferior competitors to their sensitive counterparts. We examined whether resistant mutants derived from exposure to high bacteriocin doses show growth costs. Across two independent experiments, we find that resistant cultures show significantly lower growth rates than sensitive cultures and achieve significantly lower optical densities after 24 hours of growth (Figure 4).

We find that a low bacteriocin dose did not impose strong selection for resistance, but was less effective at suppressing total densities of the target strain than the high dose (Figure 2). However, we find that incorporating a heterospecific competitor with low bacteriocin dose can allow the use of a competitor strain can allow low bacteriocin dosage to effectively reduce target cell density and significantly reduces the emergence of bacteriocin resistance. Further, a bacteriocin-producing competitor used alone can be just as effective. These results provide promising leads to suggest that using a biotherapeutic agent such as a live, heterospecific competitor may confer increased sustainability to the use of bacteriocins as alternative therapeutics. The choice of the live competitor will be the most important, and perhaps, also the most limiting factor in the application of this therapeutic approach, as the competitor cells are expected to increase in density. Currently in-use probiotic strains may be the most viable candidates, and could be further engineered to attack specific targets (reviewed in Sola-Oladokun, Culligan, & Sleator, 2017; Williams, Gebhart, Martin, & Scholl, 2008). Additional candidates may be found by screening commensal bacterial strains for specific bacteriocin activity (Lakshminarayanan *et al.*, 2013), as pathogenic strains often have non-pathogenic relatives. For example, the commensal *E. coli* G3/10 produces a bacteriocin that can inhibit enteropathogenic *E. coli* E2348/69 and has been effectively used in the treatment of irritable bowel syndrome (Zschüttig *et al.*, 2012).

In summary, we have examined whether the concept of competitive suppression of resistance can be harnessed by incorporating heterospecific competitors in antimicrobial treatment. Our *in-vitro* results suggest that the evolution of bacteriocin resistance can be

significantly suppressed by either incorporating a live, non-bacteriocin-producing competitor strain along with low bacteriocin dose, or using a bacteriocin-producing agent alone. Both these treatments also successfully suppress the overall density of target cells regardless of resistance phenotype. Our experiment provides proof-of-concept for the use of biotherapeutic agents as a template for future investigations. Conceptually, this approach should be applicable across other antimicrobials as well, and may be highly effective for treating a range of infections such as epithelial infections, including burns and wounds of the skin and mouth, as well as, gastrointestinal and lung infections, which can be recalcitrant to current therapies in immunocompromised, elderly, and cystic fibrosis patients. Future work investigating the efficacy of competitive suppression in spatially structured environments and *in vivo* models will lead to a greater understanding of the applicability of competitive suppression in treatment. In conclusion, we have provided proof-of-concept for a novel approach to impede the emergence of antimicrobial resistance, and provide a template for future investigations to explore in relevant disease settings.

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Figure 1

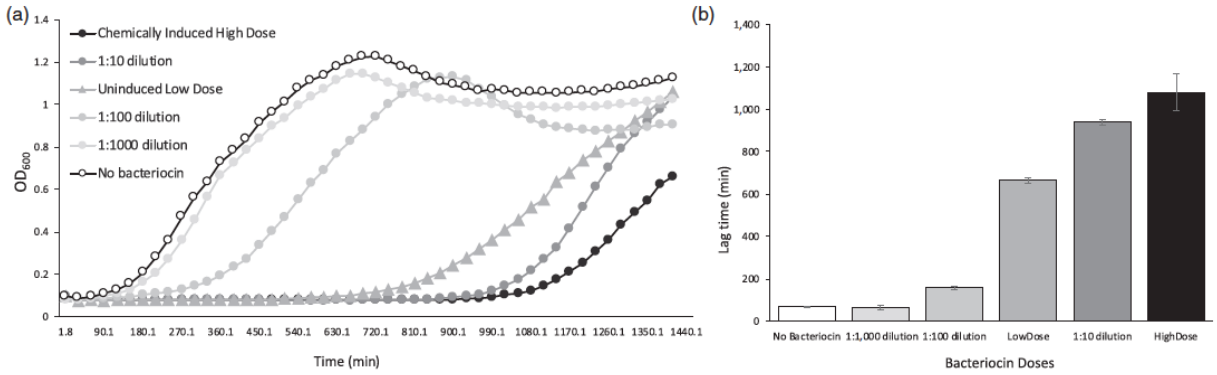

Figure 1: Bacteriocin doses measured by induced growth-inhibition of sensitive strains. (A) Growth curves of a bacteriocin-sensitive strain when exposed to different bacteriocin doses. Doses include the chemically induced 'high dose' used in this study, three consecutive 10-fold dilutions of the high dose, and the 'low dose' which was derived from chemically uninduced, 24-hr cultures of the bacteriocin producing strain. Each marker point represents an average OD_{600} value of four technical replicates. (B) Mean+/- S.E.M. lag times of the bacteriocin-sensitive cultures exposed to the different bacteriocin doses. Exposure to higher bacteriocin doses results in increased lag times due to growth inhibition.

Figure 2

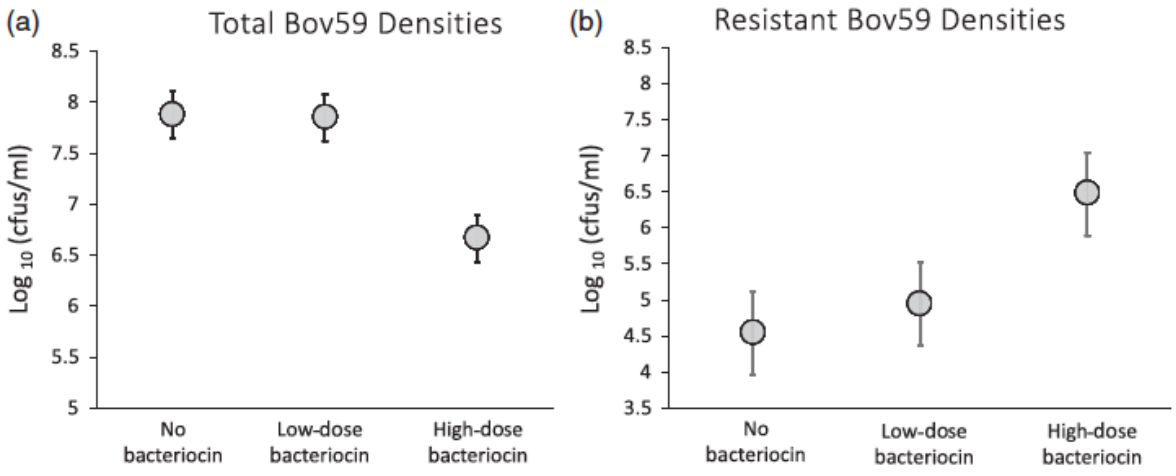

Figure 2: Effectiveness of bacteriocin dose (A) and evolution of resistance (B) in response to exposure to no, low, or high doses of bacteriocin. Total cell densities of the target strain Bov59 (A) are significantly lower in the high bacteriocin treatment as compared to low bacteriocin and negative control. However, the density of resistant Bov59 cells (B) of the target strain is significantly higher in the high bacteriocin treatment than other treatments. Data are shown as $\log_{10}$ (CFU/ml) after 24-hour exposure. Gray circles represent the mean of 9 experimental replicates with 95% confidence intervals. Detection limit for cell density counts was 10^5 CFU/ml for the 'low dose' and 'no bacteriocin' treatments, and 10^4 CFU/ml for the 'high dose' treatment.

Figure 3

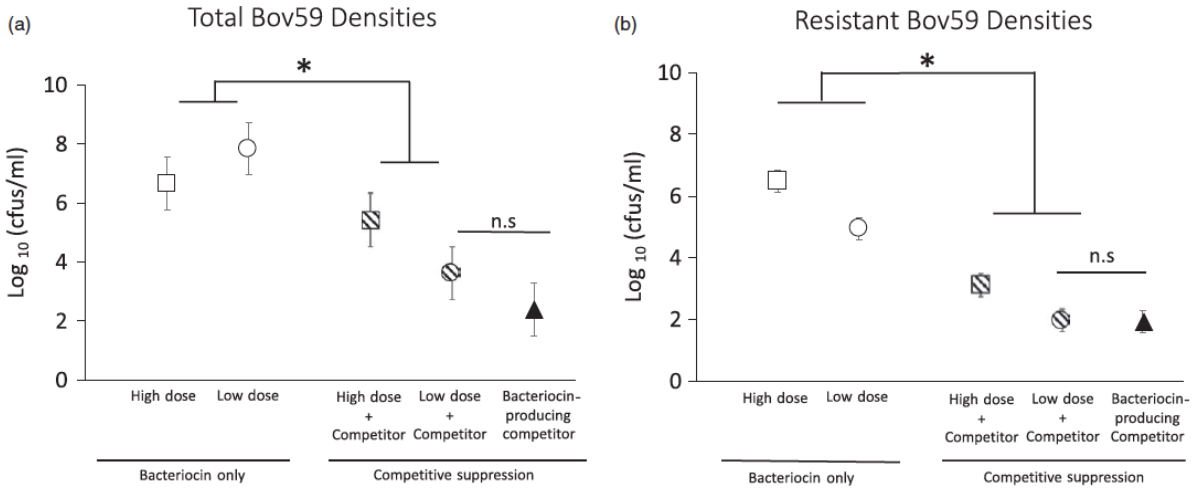

Figure 3: Suppression of total Bov59 density (A) and evolution of resistance (B) in response to bacteriocin exposure and competitive suppression. Only high and low doses of bacteriocin were administered in the bacteriocin only groups, while a live competitor was introduced in addition to bacteriocin doses in the competitive suppression group. The addition of a competitor significantly reduced the total cell densities (A) as well as resistant cell densities (B) of the target strain Bov59 at both doses of bacteriocin (* $p < 0.0001$). The combination of low dose and competitor was particularly effective at suppressing total cell densities (Dose x Competitor interaction: $F_{1,24} = 13.8$, $p = 0.001$), most likely due to the low levels of evolved resistance. Exposure to a bacteriocin-producing competitor alone was as effective as the low dose with competition treatment (n.s., $p > 0.05$). Data are shown as $\log_{10}$ (CFU/ml) after 24-hour exposure. Each symbol represents the mean of 9 experimental replicates with 95% confidence intervals for the respective treatment. Detection limit was 10^3 CFU/ml for the 'high dose + competitor' treatment and 100 CFU/ml for the 'low dose + competitor' and 'bacteriocin producing competitor' treatments.

Figure 4

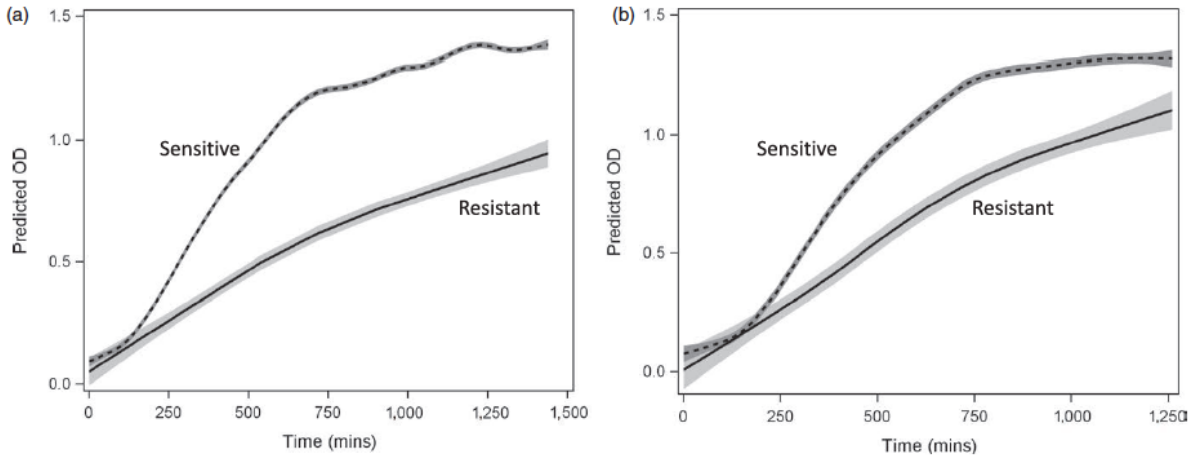

Figure 4: Cost of bacteriocin resistance as measured by reduced growth of resistant colonies (solid line, light grey shading) relative to sensitive colonies (dashed line, dark grey shading) when grown in the absence of bacteriocin. Predicted OD (+/- 95% CI of mean) for two sensitive colonies and nine resistant colonies isolated from one replicate of the main experiment are shown in (A), while growth of five sensitive and five resistant colonies isolated from an independent experiment are shown in (B).

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EDUCATION & RESEARCH APPOINTMENTS

Ph.D.	Indiana University, Evolution, Ecology, and Behavior, GPA: 4.0 Advisors: Curtis M. Lively, Farrah Bashey-Visser, July 2019
MSc. Biology	Tata Institute of Fundamental Research, Class: Distinction, Advisor: Vidita A. Vaidya, July 2013
BSc. Microbiology	University of Mumbai, Class: First, Rank: First, May 2010
Undergraduate Summer Research	Project Oriented Biological Education Fellow, Jawaharlal Nehru Center for Advanced Scientific Research, Bangalore, Advisor: Prof. Amitabh Joshi, 2008-2010

PUBLICATIONS (#Undergraduate author)

6. A. Bhattacharya, A. J. Stacy#, F. Bashey. Suppression of bacteriocin resistance using live, heterospecific competitors. *Evolutionary Applications.* ***12*****(6)**:1191-1200

5. A. Bhattacharya, H. T. Pak#, F. Bashey. 2018, Plastic responses to competition: Does bacteriocin production increase in the presence of nonself competitors? *Ecology and Evolution*. ***8*****(14):**6880–6888.

4. A. Sood*, S. Pati*, A. Bhattacharya, K. Chaudhari, V. A. Vaidya. 2018, Early emergence of altered 5-HT2A receptor-evoked behavior, neural activation and gene expression following maternal separation. *Int. J. Dev Neurosci.* ***65:****21-28*

3. S. P. Slowinski*, L. T. Morran*, R. C. Parrish, E. R. Cui, A. Bhattacharya, C. M. Lively, P. C. Phillips, 2016, Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations. *Evolution*. **70:**2632-2639

2. M. Pusalkar, D. Suri, A. Kelkar, A. Bhattacharya, S. Galande, V. A. Vaidya. 2016. Early stress evokes dysregulation of histone modifiers in the medial prefrontal cortex across the life span. *Dev Psychobiol*. **58 (2)**:198-210

1. D. Suri, A. Bhattacharya, V. A. Vaidya. 2014. Early stress evokes temporally distinct consequences on the hippocampal transcriptome, anxiety and cognitive behaviour. *Int. J. Neuropsychopharmacol*. **17**: 289–301

RESEARCH GRANTS

2016	Sigma Xi, Grants-in-Aid of Research Award ($500)
2014	Society for the Study of Evolution, Rosemary Grant Award ($2,500)

FELLOWSHIPS AND TRAVEL AWARDS

Fellowships

2019 (Spring)	Center for Integrative Study of Animal Behavior Predoctoral Fellowship
2018 (Spring)	Center for Integrative Study of Animal Behavior Predoctoral Fellowship
2017 (Summer)	Louise Constable Hoover Fellowship, Department of Biology, Indiana University
2016 (Summer)	Donna Graam Fellowship, Indiana University
2008-2010	Project Oriented Biological Education Fellowship, JNCASR, India

Travel Awards

Indiana University Provost's Women in Science Travel Award ($500-1000)	2014, 2015, 2016, 2017, 2018
Indiana University Center for the Integrated Study of Animal Behavior Travel Award ($500)	2014,2016, 2018
Indiana University Department of Biology Travel Award ($250)	2014,2015, 2016,2017, 2018

HONORS

2013	Distinction, MSc Thesis, Tata Institute of Fundamental Research, Mumbai
2010	Sir Dinshaw Manockjee Petit (First Baronet) Science Prize, University of Mumbai

PRESENTATIONS

1. Ecology and Evolution of Infectious Diseases (EEID) 2019, Princeton, NJ, USA ***[Talk]***
2. University of Iowa, Ecology and Evolution Departmental Seminar, 2018 ***[Invited seminar]***
3. Evolution, 2018, Montpellier, France ***[Contributed talk]***
4. Microbial Population Biology, GRC 2017, Andover, NH, USA ***[Poster]***
5. Evolution, 2016, Austin, TX, USA ***[Contributed talk]***
6. Ecology and Evolution of Infectious Diseases (EEID) 2016, Ithaca, NY, USA ***[Poster]***
7. Ecology and Evolution of Infectious Diseases (EEID) 2015, Athens, GA, USA ***[Poster]***
8. Microbiology Retreat, Indiana University, 2014 and 2016, Bloomington, IN, USA ***[Poster]***

TRAINING ACTIVITIES

1. Guarda Workshop in Evolutionary Biology 2014, Guarda, Switzerland

OUTREACH

2016-17	Writer and Associate Editor, ScIU - Conversations in Science at Indiana University
2016-17	Instructor, Jim Holland Summer Enrichment Program, Indiana University
2015-18	Volunteer at Indiana University's "Science Fest"
2015-16	Groups STEM Mentor for first generation college students at Indiana University

TEACHING

Teaching Appointments

2013-2017	Associate Instructor, Indiana University : L113 Introductory Biology, B200 The Intricate Human, S318 Honors Evolution, L111 Evolution and Diversity, L318 Evolution, L104 Biology of the Senses

Guest lectures

Spring 2019	Lecture: 'The evolutionary ecology of spiteful bacteriocin production in natural populations of insect-pathogenic bacteria'. A500, CISAB, Indiana University
Fall 2016	Laboratory exercise on 'Rock Paper Scissors' game to understand mechanisms that maintain biodiversity, S318, Honors Evolution, Indiana University
Spring 2016	Laboratory exercise on the evolution of antimicrobial resistance, S318, Honors Evolution, Indiana University
Spring 2016	Lecture entitled 'The evolution of social behavior' L318, Evolution, Indiana University

MENTORING

2018	Ms. Valeria C. Toro Diaz (University of Puerto Rico, Mayaguez), CISAB REU Indiana University.
2017-19	Ms. Sarah Montgomery, Undergraduate Employee, Indiana University Mr. Alexander J Stacy, Undergraduate Employee, Indiana University
2016 – 17	Ms. Elizabeth Lambert, Emerging Scholars REU Women Program, Indiana University
2016	Ms. Elizabeth Huls, High School Research Student, Indiana
2015-2016	Ms. Hannah Pak, Undergraduate Employee, Indiana University
2014-2015	Mr. Cheyenne Smith, Undergraduate Volunteer, Indiana University Mr. Jay Trivedi, Undergraduate Volunteer, Indiana University

PROFESSIONAL SERVICE

Peer review

2016	Molecular Ecology

Organizing conferences, moderating panel discussions

2016	Session Moderator, IU Center for the Integrative Study of Animal Behavior (CISAB) Conference
2015-2017	IU CISAB Animal Behavior Conference, Poster Committee
2012	International Society for Developmental Neuroscience, Annual Meeting, Mumbai, Volunteer

PROFESSIONAL SOCIETY MEMBERSHIPS

American Society of Naturalists
Society for the Study of Evolution
Indiana University Center for the Integrative Study of Animal Behavior